THE EFFECT OF AUTOCLAVING ON THE NUTRITIVE VALUE OF EDESTIN [1]

HARRY A. WAISMAN [2] AND C. A. ELVEHJEM

Department of Agricultural Chemistry, University of Wisconsin, Madison

TWO FIGURES

(Received for publication February 9, 1938)

During studies on the preparation of rations low in certain heat labile factors it was observed that the growth of rats placed on a natural grain ration, which had been autoclaved for 5 hours at 15 pounds pressure and 120°C., was retarded in spite of the addition of vitamin B_1 and factor W. This observation made it evident that further studies on the heat treatment of experimental rations were necessary. The work to be reported in this paper was started mainly to investigate the effect of autoclaving upon the nutritive properties of proteins. For this purpose the protein, edestin, was used in most of the work and the heat treatment was limited to autoclaving.

Geiling ('17) obtained poor growth in mice when he used a ration containing 10% casein which had been autoclaved for 1 hour at 15 pounds pressure. Today we know that this level of casein is suboptimal and that the rest of the ration was undoubtedly deficient in other factors. McCollum and Davis ('17) also used autoclaved casein at a level of 10% and found that growth was inhibited. Komatsu and Okinaka ('26) in studies on the effect of 'superheated water' on proteins, found that edestin treated under these conditions underwent a profound change. The groups containing amide N

[1] Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

[2] National Live Stock and Meat Board Fellow.

were removed from the protein molecule at the beginning of the reaction while those containing diamino N were removed more slowly. Other work on the effect of heat treatment on the biological value of proteins has been done by Morgan and Petro ('30) and Marletta ('29) and they noted that growth was retarded when the protein was subjected to autoclaving. Salmon ('32) found that casein heated at 130°C. for 48 hours and fed as the sole source of protein did not support growth even when vitamins B_1 and B_2 (G) were supplied by a protein free extract of yeast. Hogan ('17) found that autoclaving had no effect on the nutritive value of egg white, even after 6 hours at 30 pounds pressure. Morgan and King ('26) observed that autoclaving cereal grains decreased the biological value of the proteins contained in these grains. Mitchell ('35) found that lysine in milk was destroyed in the later stages of the steam roller process for the manufacture of commercial dry skim milk powder. Voegtlin and Thompson ('36) observed that administration of lysine to a diet prepared with 30% whole milk powder (autoclaved for 1 hour at 15 pounds pressure) removed the inhibition of normal and malignant growth in mice. Block et al. ('34) found that the same amounts of lysine could be isolated from casein before and after heating at 150°C. for 65 minutes.

EXPERIMENTAL

The edestin used in our rations was isolated, according to the method of Osborne (1892), from the fiber hemp seed. The edestin was recrystallized so that the dried crystalline material contained only traces of phosphorus as determined by the micro method of Fiske and Subbarow ('25). The animals used in our experiments were albino rats, which were started on the experimental diet at weaning, usually at 21 to 28 days of age. The relative growth rates were observed for a period of 6 to 8 weeks.

In order to study the effect of autoclaving upon edestin, it was first necessary to reappraise the nutritive value of the protein from the standpoint of our present knowledge of

accessory food factors. In devising a diet for the appraisal of the nutritive value of recrystallized edestin, we used dextrin, peanut oil, and cod liver oil, together with a mixture of salts used in this laboratory and designated as salts I ('36 a). The peanut oil furnished a source of B_4 as shown by early work in this laboratory ('36 b) and also supplied the essential unsaturated fatty acids. The cod liver oil supplied the fat soluble vitamins A and D. Besides these ingredients, some diets to be described below contained either bakers' yeast or brewers' yeast [3] together with liver extract,[3] which is the dried water soluble fraction of whole liver. When autoclaved

TABLE 1

Composition of rations

CONSTITUENT	501	504	505	507	513	539	605
Edestin	18	18	18	18	18	24	
Dextrin	68	64	68	66	67.5	61.5	68
Salts I	5	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2	2
Peanut oil	5	5	5	5	5	5	5
Bakers' yeast	2	4		2			
Brewers' yeast					0.5	0.5	0.5
Liver extract		2	2	2	2	2	2
Crude casein							18

edestin was used, the protein was placed in pans in layers about ¼ inch deep, and autoclaved for 5 hours at 15 pounds pressure and at a temperature of 120°C. The composition of each ration is listed in table 1.

The first ration tried contained edestin plus 2% of bakers' yeast as the sole source of the water soluble vitamins. The growth curves for rats receiving the ration with unautoclaved and autoclaved edestin are given in figure 1. The letter 'A' following the ration number denotes that it contains autoclaved edestin. The number of rats used for each ration varied considerably, but the number was never less than

[3] We are indebted to Dr. D. Klein for a generous supply of liver extract and to Dr. H. Levine for a supply of brewers' yeast.

four and more often four to eight were used. In order to save space, typical growth curves for one male and female rat are given for each ration. The growth on either ration 501 or 501-A did not approach that obtained on a complete ration, but the rate of growth on the ration containing autoclaved edestin was significantly lower than that obtained on the ration with the unautoclaved protein. After 4 weeks

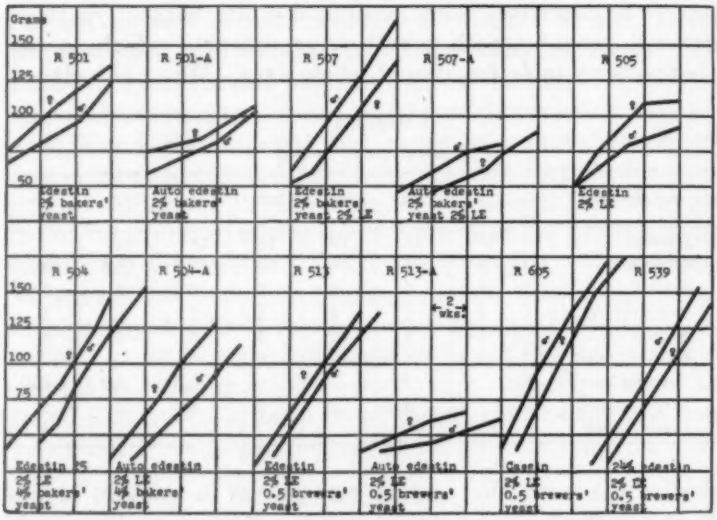

Fig. 1 Typical growth response of one male and one female rat on rations prepared with various supplements to autoclaved or unautoclaved edestin. The letter 'A' following the ration number denotes that it contains autoclaved edestin.

the level of bakers' yeast in both diets was increased to 4% of the ration. There was some growth response in the group on the unautoclaved edestin, but no response was obtained in the group receiving the heated protein. Although the increased quantity of yeast furnished some protein, the slight response in growth on the untreated protein was no doubt due to other factors. Accordingly, the next ration tried was one which contained 2 parts of liver extract together with 2% of the

bakers' yeast. Growth was sharply arrested again on the autoclaved protein (ration 507-A), while the group which received the unautoclaved protein gave good growth (ration 507). When the rats were placed on a ration which had the unautoclaved edestin and 2% liver extract but no yeast (ration 505), growth was unsatisfactory, and this is understandable in view of the limited quantity of vitamin B_1 in the ration. The next series was started in an attempt to demonstrate the effect of autoclaved protein on growth even when 2% liver extract and 4% bakers' yeast were in the diet. Again growth was superior on the unautoclaved edestin (rations 504 and 504-A). However, because of the additional protein furnished by the 4% bakers' yeast, growth on the autoclaved ration was better than that obtained when 2% yeast was used in the same ration.

Since growth responses were consistently better on the unautoclaved than on the autoclaved edestin regardless of the quantity of yeast used, it was believed that sharper differences could be obtained if the protein furnished by the yeast was reduced to a minimum without limiting the vitamin B_1 intake. A more potent source of vitamin B_1 is brewers' yeast and when this was fed at a level of 0.5% of the ration with autoclaved or untreated protein, growth was markedly inhibited on the former. (Compare rations 513 and 513-A, fig. 1). This basal ration therefore, was used in the remainder of the experiments.

To complete our data on the nutritive value of edestin, we next substituted casein for edestin in the basal ration in order to have a basis for comparing the two proteins. The growth curve is shown in figure 1, ration 605. Since the 18% casein ration produced better growth than the 18% edestin, a diet which had 24% edestin was next fed in an attempt to obtain growth comparable to that on the 18% casein. The curve for ration 539 indicates that growth on the 24% edestin was slightly better than on the 18% level of edestin, but did not reach that obtained with 18% casein.

Since the evidence is convincing that autoclaving reduces the nutritive value of the protein, it was necessary to determine whether the suppression of growth was due to an unavailability of the protein, or whether there was destruction of an amino acid indispensable for normal growth. For this purpose various supplements were added to the basal 513-A. In view of the literature cited above, it appeared as though the dibasic amino acids might have been affected, and so the first supplement tried was a basic amino acid fraction obtained from casein. The crude commercial casein was hydrolyzed with 20% hydrochloric acid for 36 hours and after removal of the excess acid, the hydrolysate was treated with activated charcoal in order to decolorize the solution. After filtration of the humin and charcoal, the hydrolysate was neutralized and a concentrated solution of phosphotungstic acid, $2H_3PO_4 \cdot 20WO_3 \cdot 25H_2O$, was added until no further precipitation of the basic amino acids occurred. The phosphotungstic acid was removed from the amino acids-phosphotungstic complex with baryta, and the solution of the basic amino acids was acidified, concentrated, decolorized, and dried in vacuo. The addition of 1 part of basic amino acids equivalent to 5% casein gave a sharp rise in growth when added to the basal diet. The average weight difference between the two groups was 56 gm. at the end of 6 weeks. (See group 1, fig. 2).

Since a response in growth was obtained with the basic amino acid fraction from casein, the indication was that one or more amino acids were concerned. The addition of 1 part of cystine [4] to the basal ration produced only a slight increase in growth, being clearly less than when the basic amino acid fraction of casein was used. There was also no response in growth when either arginine carbonate [5] or histidine hydrochloride [6] were added (groups 2, 3, 4, fig. 2). The group receiving the histidine hydrochloride was given 1% of cystine

[4] A commercial preparation.

[5] Arginine carbonate was prepared according to the procedure of Kossel and Gross ('24).

[6] A commercial preparation.

and 1% arginine after 6 weeks. There seemed to be a slight response, but not enough to warrant the conclusion that a combination of cystine and arginine was the limiting factor in our basal ration.

The only amino acid of the basic amino acid fraction that remained to be considered as being affected by autoclaving was lysine. The lysine used as the supplement to the basal ration was isolated from casein, according to the method of

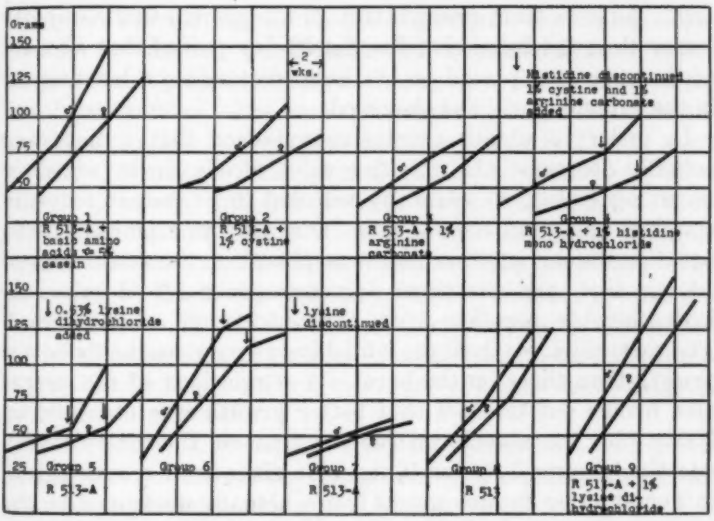

Fig. 2 Typical growth responses with various supplements added to the basal ration 513-A.

Vickery and Leavenworth ('28). The crude lysine picrate, obtained in the usual way was recrystallized and melted with decomposition at 250 to 252°C. (uncorrected). Crystalline lysine was obtained after the picrate was decomposed and the picric acid removed. A preliminary experiment was begun in which one male and one female rat were kept on the basal diet for 4 weeks, both rats increasing in weight from 38 to 55 gm. Upon the addition of 0.53% of d-lysine dihydrochloride

to the basal diet, the growth increment in 13 days was 55 to 80 gm. for the male, and 55 to 101 gm. for the female. (See group 5, fig. 2.) This marked growth indicated that lysine was the limiting factor in our basal diet. The somewhat slower growth of the male led us to believe that the level of lysine was too low for 0.53% of d-lysine dihydrochloride represented only 0.35% of the free amino acid. Accordingly, the next diet fed was the basal plus 1% of the d-lysine dihydrochloride which actually furnished 0.67% of the free amino acid. As seen from group 6 (fig. 2) the growth was distinctly better than the basal diet for the 32-day period, but as soon as the rats were placed on the basal diet alone, a leveling off in the growth curve was observed.

In order to obtain conclusive evidence that autoclaving actually decreased the nutritive value of edestin by somehow involving lysine, we carefully selected three pairs of evenly matched male and female rats. One pair was placed on the basal diet alone, another pair was placed on the unautoclaved edestin diet, and the third pair was given 1% d-lysine dihydrochloride together with the autoclaved edestin basal. The rats which received the added lysine gave markedly better growth than those on the basal. A comparison of the curves also brings out the fact that better growth was obtained on lysine plus the autoclaved edestin, than on the unautoclaved edestin (groups 7, 8 and 9, fig. 2). Since we have as yet no information on the amount of lysine actually destroyed by the heat treatment of edestin, it is plausible that 1% lysine dihydrochloride furnishes ample of this essential amino acid for good growth. When edestin is fed at a 24% level, the average growth is essentially that obtained on the basal plus 1% d-lysine dihydrochloride. The average growth of the former after 6 weeks is 144 gm. compared to 147 gm. for the latter after the same period.

DISCUSSION

Some 25 years ago, Osborne and Mendel ('12) determined the comparative nutritive value of certain proteins by noting their influence on growth. They used casein, edestin, egg albumin, and gliadin as well as other easily isolated animal and vegetable proteins. When fed for a 6-week period, the rats receiving 16.7% edestin weighed 106 gm. while those fed 11.2% weighed 86 gm. and those receiving 9% of the protein weighed only 70 gm. In another experiment these workers obtained comparable growth on diets containing either edestin or casein. Our results show that somewhat better growth was obtained when casein replaced the edestin in the basal ration used. The average weight of the animals on the casein diet after 5 weeks was 160 gm. which is 25 gm. more than the average of the animal on the edestin diet. It is possible, therefore, to demonstrate that edestin is not as complete a protein as casein when a greater effort is made to supply all the water soluble vitamins. The possibility that casein supplies additional growth factors which are not present in edestin should not be overlooked but the result with added lysine and the higher levels of edestin indicate that the limiting value of edestin is the low amount of lysine in the original preparation of edestin.

Although the present work indicates that 18% of edestin in a diet of a rat does not supply the optimum amount of all the essential amino acids, the actual growth of the rats is considerably higher than that reported by the earlier workers. We obtained a growth of 140 to 160 gm. in 6 weeks on a diet containing 18% edestin supplemented with 2% liver extract and 0.5% yeast. In Osborne and Mendel's studies, the best growth on an edestin diet was 120 gm. or less after 6 weeks. Sure ('22) obtained very poor growth on a ration of edestin 18 parts, agar agar 2 parts, butterfat 7 parts, salts 4 parts and dextrin 69 parts which carried an alcoholic extract of ether extracted wheat embryo. After 6 weeks four of the male rats weighed 90 gm. or less (two of them weighed only 60 gm.) and two of his female rats weighed approximately

120 gm. In light of our present knowledge of the factors in the B complex, it is very likely that an extract of wheat germ similar to the one used would not carry an adequate amount of all the essential factors.

The basal ration used in our work may be criticized in that both the liver extract and yeast carry definite amounts of protein but in spite of this difficulty it is apparent that the nutritive value of edestin is markedly affected, when the protein is autoclaved for 5 hours at a pressure of 15 pounds and a temperature of 120°C.

The experimental observation that lysine has the ability to fortify autoclaved edestin for good growth finds added support from quantitative figures. The quantity of lysine supplied by the 18% level of the edestin is 0.396 gm. (Mitchell and Hamilton, '29). When in addition 1% d-lysine dihydrochloride is fed, there is furnished an additional 0.672 gm. of the free amino acid. This totals to 1.068 gm. It is conceivable that upon autoclaving edestin, a good part of it is either destroyed or rendered unavailable. When autoclaved protein is fed at a 24% level, there is ostensibly furnished 0.53 gm. of lysine. If we assume that all of the lysine is destroyed on autoclaving then the addition of 1% of d-lysine dihydrochloride furnishes 0.67 gm. and is greater than the 0.53 gm. furnished by the 24% level. The growth response with the 1% addition of lysine dihydrochloride is better than the 24% level of untreated protein, thus bearing out the contention that higher levels of lysine will produce increments in growth until the optimum level is reached. The results emphasize the difficulties that are still evident in the study of proteins in a synthetic diet. Although the 18% casein produced better growth than the 24% level of edestin, we cannot say definitely that the result can be ascribed to either the protein value of the casein, or to the unknown factors carried along by this material in the process of its isolation.

SUMMARY

1. Autoclaving the protein, edestin, markedly alters its nutritive value as shown by retarded growth on a complete diet.

2. The limiting factor in autoclaved edestin has been shown to be lysine.

3. The reappraisal of the nutritive value of edestin in the light of our present knowledge of accessory food factors is discussed.

LITERATURE CITED

BLOCK, R. J., D. BREESE-JONES AND C. E. F. GERSDORFF 1934 Effect of dry heat and dilute alkali on lysine content of casein. J. Biol. Chem., vol. 105, p. 667.

FISKE, C. N., AND Y. SUBBAROW 1925 Colorimetric determination of phosphorus. J. Biol. Chem., vol. 66, p. 375.

GEILING, E. K. 1917 Nutritive value of diamino acids occurring in proteins for maintenance of adult mice. J. Biol. Chem., vol. 31, p. 173.

HOGAN, A. G. 1917 High temperatures on nutritive value of foods. J. Biol. Chem., vol. 30, p. 115.

KLINE, O. L., H. R. BIRD, C. A. ELVEHJEM AND E. B. HART 1936 An improved synthetic ration for vitamin B_4 studies. J. Nutrition, vol. 11, p. 515.

KLINE, O. L., C. A. ELVEHJEM AND E. B. HART 1936 Further evidence for the existence of vitamin B_4. Biochem. J., vol. 30, p. 780.

KOMATSU, S., AND C. OKINAKA 1926 Studies on proteins. II. Action of superheated water on proteins. Bull. Chem. Soc. Japan, vol. 1, p. 102.

KOSSEL, A., AND R. G. GROSS 1924 Über die darstellung und quantitative bestimmung des arginins. Zeit. f. Physiol. Chem., Bd. 135, S. 167.

MCCOLLUM, E. V., AND M. DAVIS 1917 Cause of loss of nutritive efficiency of heated milk. J. Biol. Chem., vol. 23, p. 247.

MARLETTA, A. 1929 Food value of casein and albumin treated in an autoclave with high temperature and in an alkaline medium. Biochem. terap. sper., vol. 16, p. 297 and C. A. Vol. 24, p. 5804.

MITCHELL, H. H. 1935 Nutritive values of dried skim milk. Forty-seventh Annual Report, Illinois Agric. Exp. Station, p. 82.

MITCHELL, H. H., AND T. S. HAMILTON 1929 The biochemistry of the amino acids. 1st ed., pp. 190–192. Chemical Catalog Company, New York City.

MORGAN, A. F., AND A. F. PETRO 1930 Effect of heat treatment upon biological value of proteins. J. Home Econ., vol. 22, p. 857.

MORGAN, A. F., AND F. B. KING 1926 Changes in biological value of cereal proteins due to heat treatment. Proc. Exp. Biol. and Med., vol. 23, p. 353.

OSBORNE, T. B. 1892 Crystallized vegetable proteins. Am. Chem. J., vol. 14, p. 662.

OSBORNE, T. B., AND L. B. MENDEL 1912 Beobachtungen über wachstum bei Futterungsversuchen mit isolierten nahrungs substanzen. Zeit. f. Physiol. Chem., Bd. 80, S. 307.

SALMON, W. D. 1932 Effect of heating casein on gain of rats. Alabama Agr. Exp. Sta., Ann. Reports, no. 43.

SURE, B. 1922 Nutritive value of edestin Cystine and lysine as growth limiting factors in that protein. Am. J. Phys., vol. 61, p. 1.

VICKERY, H. B., AND C. S. LEAVENWORTH 1928 Basic amino acids of horse hemoglobin. J. Biol. Chem., vol. 79, p. 377.

VOEGTLIN, C., AND J. W. THOMPSON 1936 Lysine and malignant growth. 1. Amino acid lysine as a factor controlling the growth rate of a typical neoplasm. Public Health Reports, vol. 51, p. 1429.

THE EFFECT OF AMINO-ACID SUPPLEMENTS AND OF VARIATIONS IN TEMPERATURE AND DURATION OF HEATING UPON THE BIOLOGICAL VALUE OF HEATED CASEIN

ETHELYN O. GREAVES, AGNES FAY MORGAN AND MINNETTE K. LOVEEN

Laboratory of Household Science, University of California, Berkeley

TWO FIGURES

(Received for publication February 5, 1938)

It was shown in this laboratory (Morgan and King, '26; Morgan, King, Boyden and Petro, '31) that dry heating of cereal proteins and casein at 200° and at 150° for 30 to 45 minutes materially depressed the biological value of these proteins as determined by growth and nitrogen balance studies on rats.

This finding has been confirmed by Boas-Fixsen and Jackson ('32) for casein heated at 112°, by Kon and Markuze ('31) for the crust protein of rye bread, by Chick, Boas-Fixsen, Hutchinson and Jackson ('35) for casein heated at 150° for 66 hours and lactalbumin for 72 hours at 120°, by Fairbanks and Mitchell ('35) for the proteins of dried skim milk, and by Seegers and Mattill ('35 a, b) for the proteins of beef round, heart, kidney and liver heated at 100° to 130° for prolonged periods. Morgan and Kern ('34) also found loss of nutritive value in meat proteins due to cooking.

The present paper reports two further studies on this question, 1) the results of supplementing the heated casein diet with certain amino acids,[1] and 2) an attempt at the determination of the critical temperatures and time of heating which produce measurable effects upon the biological value of the casein.[2]

[1] A preliminary announcement of part of these data has been published (Greaves and Morgan, '34).

[2] Assistance in the latter part of this investigation was rendered by Works Progress Administration Project No. 2445, assigned to the University of California.

PART I

The effect of amino-acid supplements

Even though no assumptions be made as to the nature of the damage it seemed worth while to attempt to discover which if any of the essential amino acids are most affected by the heating. It could not be assumed that only one amino acid was affected, nor even that only one group was affected, since varying temperatures and times of exposure to heat had been found to exert varying degrees of damage upon the nutritive value of proteins and particularly of milk proteins. It seemed possible that one amino acid or group of amino acids might be first affected and that different proteins might appear to have different sensitivities to heat in proportion to the amount or the molecular exposure of such heat-sensitive amino acids. Secondary damage resulting from further heat treatment might be due to attack upon another amino acid or group of amino acids, larger in amount or better protected by their placing in the molecule. The latter group of amino acids might in another protein be first attacked. In the case of casein the vulnerable points are cystine because of paucity and the dibasic acids possibly because of position.

Methods

The same type of casein was used in all the experiments here reported, an acid-precipitated commercial product hereafter designated raw casein, and the same heated for 30 minutes at 140° in layers ½ inch deep in a thermostatically controlled electric oven. The nitrogen content of the raw casein was 13.5% and of the heated casein 14.2%.

The growth experiments were made with young rats 28 days old at the beginning, fed a basal diet made up of the casein 12 to 22% (9 to 18% protein), agar 4, Osborne and Mendel salt mixture 4, Crisco 20, and cornstarch to make 100. Cod liver oil 2 drops and Harris yeast vitamin extract 100 mg., were fed separately to each animal daily as sources of vitamins.

The nitrogen balance experiments were carried out as previously described (Morgan, King, Boyden and Petro, '31), using eight mature male and eight mature female rats. Each of the sixteen rats was submitted in turn to a balance study with various diets containing the casein, raw, heated, and each of these supplemented with 0.2% of the diet as d-lysine dihydrochloride, l-cystine, and l-tyrosine. There were thus thirty-two determinations of biological values for each of the caseins, heated and raw, alone and supplemented by the three amino acids studied. In order to save space the full data are

TABLE 1

Digestibilities and biological values of raw and heated (at 140° for 30 minutes) casein with and without lysine, tyrosine and cystine (thirty-two determinations on each diet)

CASEIN	AVERAGE CORRECTED DIGESTIBILITY	AVERAGE BIOLOGICAL VALUE	DECREASE FROM RAW CASEIN VALUE	
			Digestibility	Biological value
			%	%
Raw	97±0.5	69±0.3		
Heated	93±0.5	57±0.4	4	17
Raw plus lysine	97±0.4	67±0.3		
Heated plus lysine	90±0.5	64±0.6	7	4
Raw plus tyrosine	96±0.4	67±0.2		
Heated plus tyrosine	87±0.7	57±0.4	9	15
Raw plus cystine	92±0.6	77±0.5		
Heated plus cystine	87±0.6	66±0.4	5	14

not presented but a summary of the biological values and digestibilities is given in table 1. In each case the heated casein supplemented by the amino acids is compared with the corresponding raw casein-supplemented diet.

The unsupplemented heated casein was found to have a biological value of 57 as compared with 69 for the raw, a loss of 17%, although the digestibility was lowered by only 4%. With tyrosine added the digestibility of the heated diet was decreased but the biological value was little changed. Apparently tyrosine is not concerned in the heat damage produced by the procedure used.

When lysine was added the digestibility of the heated casein was slightly decreased but the biological value was definitely raised from 57 to 64. In seven of the rats the biological value of the lysine-supplemented casein was equal to or better than that of the comparable raw diet, in seven the improvement was definite but not complete, and in two no improvement was shown. It must be concluded that the lysine of the heated casein had been injured.

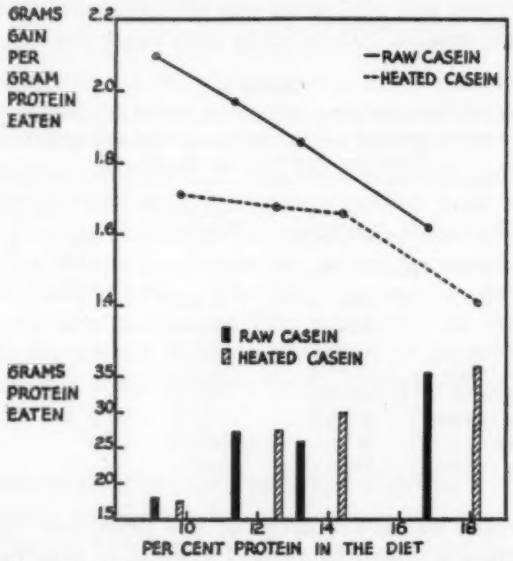

Fig. 1 The gain in weight per gram of raw and heated casein eaten in 28 days, fed at varying levels in the diet (eight to thirteen rats in each group).

When cystine was added, uniform increase in the biological values of both the raw and heated casein resulted but the difference between them remained unchanged, 77 and 66, as compared with the unsupplemented values, 69 and 57. Clearly the cystine of the heated casein was not damaged.

The growth studies were continued for 28 days in all cases. The effect of variation in level of both raw and heated caseins is shown in figure 1. In spite of slightly greater intake of the

heated casein at each level, the growth per gram of protein eaten was uniformly less for these groups. The maximum gain for both raw and heated diets was seen at the 9 to 10% level at which level also the margin of better growth in proportion to protein eaten shown on the raw diet was greatest. At all higher levels, however, the raw casein proved definitely superior to the heated. These relations of protein level to growth were similar to those previously reported in a study of raw and toasted wheat gluten (Morgan, King, Boyden and Petro, '31).

For the amino-acid supplemented diets both 9 and 12% levels were used for the growth studies (table 2). Supplements of l-tryptophane and l-tyrosine were found to have no effect upon the growth value of either raw or heated casein and these amino acids may therefore be said to be unaffected by this amount of heating. Supplements of either 0.1% or 0.2% cystine improved the growth obtained with both raw and heated casein equally, about 15%. Lysine improved the growth on the heated diet but depressed slightly that on the raw, resulting in a difference of only 5% in favor of the latter. One group fed the heated casein with addition of both lysine and cystine showed growth fully as good as that of the raw casein group.

L-histidine di-hydrochloride was used to supplement the 10% heated and raw casein diets. Although food intakes of the groups to be compared were more diverse than usual, improvement in growth performance on the heated diet when thus supplemented was evident although in smaller degree than had been produced by the lysine supplement. Approximately two-thirds of the injury was remedied by the lysine, less than one-half by the histidine. In a later experiment with casein heated at 130° instead of 140° histidine was used as supplement to the 12% diet. The damage due to heating at 130° for 30 minutes was found to be less than that at 140°, being usually a reduction of 10 to 14% of growth value instead of 17 to 20%. There was no supplementing effect of the histidine at this level and with this amount of heat damage. Whether

TABLE 2

Growth value of raw and heated (at 140° for 30 minutes) casein at 9 to 12% level, with and without added amino acids

DIET	PROTEIN LEVEL IN DIET	NUMBER OF RATS	DAILY FOOD INTAKE	TOTAL PROTEIN INTAKE IN 28 DAYS	AVERAGE INITIAL WEIGHT	GAIN IN 28 DAYS	GAIN PER GRAM OF PROTEIN EATEN	DECREASE IN GROWTH VALUE
	%		gm.	gm.	gm.	gm.	gm.	%
Raw casein	9.1	9	6.9	17.6	44	37	2.10	
Heated casein	9.9	9	6.0	16.9	44	29	1.71	19
Raw plus 0.2% tryptophane	9.1	5	7.7	19.6	59	35	1.78	
Heated plus 0.2% tryptophane	9.9	5	7.2	21.0	59	30	1.43	19
Raw plus 0.2% lysine	9.1	6	8.1	20.7	60	41	2.00	
Heated plus 0.2% lysine	9.9	6	8.0	22.1	60	42	1.90	5
Heated plus 0.1% lysine and 0.1% cystine	9.8	8	7.6	20.7	55	44	2.13	0
Raw plus 0.2% histidine	9.0	5	7.8	19.6	58	42	2.14	
Heated plus 0.2% histidine	9.9	5	8.1	22.4	58	44	1.97	8
Raw casein	11.3	12	8.7	27.4	58	54	1.97	
Heated casein	12.5	23	7.8	27.0	55	44	1.63	17
Raw plus 0.2% tyrosine	11.3	12	8.7	27.4	58	54	1.97	
Heated plus 0.2% tyrosine	12.5	10	7.9	27.4	57	46	1.68	15
Casein heated in vacuo	12.5	13	7.6	26.6	52	43	1.61	18
Raw plus 0.1% cystine	11.5	14	8.3	26.6	52	60	2.25	
Heated plus 0.1% cystine	12.4	13	7.7	26.7	51	52	1.94	14
Raw plus 0.2% cystine	11.6	12	8.3	26.9	60	62	2.30	
Heated plus 0.2% cystine	12.4	16	8.1	28.0	56	55	1.96	15
Raw plus [1] 0.2% histidine	12.0	5	9.4	31.7	60	74	2.35	
Heated plus [1] 0.2% histidine	12.0	8	8.5	28.5	56	60	2.10	10
Raw casein [1]	12.0	8	8.9	29.9	50	71	2.37	
Heated casein [1]	12.0	9	8.7	29.3	48	62	2.11	11

[1] From another series in which casein was heated at 130° instead of 140° for 30 minutes, table 3, series 3.

this means that injury to the histidine occurs only after more severe heating than does that to the lysine cannot be established from the second experiment since the lysine supplement was not used in that series.

One lot of rats was fed a specially heated casein prepared by heating in vacuo at 140° for 30 minutes (table 2). The reduction in growth value was found to be nearly the same, 18%, as for the oven-heated product which showed 17% loss of growth value at the same level of intake. This appears to establish the fact that the injury is not primarily due to oxidation.

Although lysine is well represented in casein, it was undoubtedly damaged during heating at 140°. Histidine, also present in casein in adequate amounts was damaged at 140° but apparently not at 130°.

The improvement by cystine supplement was just as marked on the raw as on the heated casein diet. The conclusion of Fairbanks and Mitchell ('35) that the first damage to heated skim milk proteins is to the cystine was based on the supplementing of mildly heated dried milk with cystine, apparently without parallel supplementing of the raw skim milk. It seems likely that the latter proteins would also show increased growth value with added cystine. More severely heated milk, however, responded only to additions of lysine since the damaged lysine had become the limiting factor now, instead of the cystine as had probably been the case in the less scorched milk powder as well as in the raw milk.

It would appear that the first damage caused by heat to casein, as well as to wheat gluten, is to the lysine and the next is to the histidine. Tryptophane, tyrosine and cystine are not affected. The leucines, phenylalanine, valine, threonine and a sufficient margin of methionine must also be left in the heated proteins if these are accepted as finishing the list of essential amino acids.

Failure in digestibility must account for part of the damage but probably not for all since the discrepancy between lowering of digestibility and of biological value is so great. Both

raw and heated casein for instance when supplemented by cystine showed greatly decreased digestibility but, at the same time, decidedly improved biological values. The addition of tyrosine depressed digestibility and biological value in both raw and heated proteins. The addition of lysine slightly lowered digestibility in raw and heated caseins while decidedly increasing biological value in the heated only. Selective absorption of certain amino acids due to failure in digestion of the heated protein as suggested by Seegers and Mattill ('35 b) may represent the facts, but the reason for the failure of lysine to be absorbed from heated protein must still be sought. Is it because the lysine is present in the intestine in indissoluble combination with something else or because it has undergone a change such that lysine is no longer present although its nitrogen is still absorbable? The latter suggestion appears to fit the facts of the nitrogen balance studies here reported.

PART II

The effect of variations in temperature and time of heating upon the nutritive value of casein

Since several reports had indicated that the amount of change produced in the nutritive value of proteins by heating varied with the temperatures and duration of heating periods a series of growth experiments with rats was planned to test the extent of these changes in casein.

The diet in all cases contained 12% casein (nitrogen 1.92%). This level was chosen since it had been found (fig. 1) that the best gains per gram of protein eaten were obtained at that level but the rate of growth remained well below the maximum.

A series of animals was fed with casein samples heated at 100°, 120° and 130° for varying periods, 30 minutes, 2 hours, 8 hours and 24 hours. The data are given in table 3. The loss in growth value was seen to be progressively greater as the interval of heating at 130° was increased, 11, 18, 34 and 39% of the value shown by the raw casein used in this group. Heating at 120° caused perceptible damage only if continued for 8 hours, but this damage was much increased by extending the heating for 24 hours.

TABLE 3

The effect upon its nutritive value of washing casein and of heating it at 100°, 120° and 130° for varying periods

SERIES	TEMPERATURE OR TREATMENT	PERIOD OF HEATING	NUMBER OF RATS	INITIAL WEIGHT	GAIN IN 28 DAYS	TOTAL PROTEIN INTAKE	GAIN PER GRAM PROTEIN EATEN	DECREASE IN GROWTH VALUE
		hours		*gm.*	*gm.*	*gm.*	*gm.*	%
1	Raw		15	56	64	27.8	2.29±0.07	
	100°	0.5	7	57	57	25.2	2.26±0.07	None
		2	7	51	62	26.2	2.36±0.04	None
		8	6	53	65	28.8	2.26±0.06	None
	Raw washed		9	53	51	26.0	1.96±0.06	15
	Boiled at 100°	0.5	8	53	53	27.9	1.90±0.03	17
		2	9	55	56	26.7	2.09±0.06	9
	120°	0.5	10	67	60	26.0	2.30±0.06	None
		2	9	52	65	29.0	2.24±0.05	None
		8	18	52	53	26.6	2.00±0.04	14
2	120°	24	6	50	41	23.0	1.78	27
	Raw		6	53	58	23.6	2.45	
3	Raw		8	54	71	29.9	2.37±0.03	
	130°	0.5	9	48	62	29.3	2.11±0.04	11
		2	15	52	59	29.4	2.01±0.05	18
		8	9	49	44	28.2	1.56±0.05	34
		24	11	50	41	28.9	1.42±0.03	39
4	Raw		8	50	71	28.8	2.46±0.06	
	Raw washed		7	55	55	28.0	1.97±0.03	20
	Raw plus filtrate from heated (130°) casein		6	55	70	28.8	2.43±0.05	None
	130°	8	7	53	37	27.2	1.36±0.01	45
	130° washed	8	10	54	39	28.4	1.39±0.03	43

The effect of boiling at ordinary atmospheric pressure was tried but was necessarily compared with that of cold water washing as well as with dry heating at 100°. The casein was soaked in the same volume of cold distilled water for the same length of time which was used for the boiling experiments. It was then filtered and air-dried before a fan. A decided loss of growth value was found to result as shown in table 3, series 1. This experiment was repeated later in another connection and as shown in table 3, series 4, again resulted in loss of growth value. Loss of accessory food factors can hardly account for the decrease in view of the obvious adequacy of the water-soluble vitamin supplement given in all cases. It seems unlikely that enough lactalbumin could be retained by the raw casein to increase its biological value in the amount indicated even assuming the superior value of lactalbumin reported by Osborne and Mendel ('16). Boiling the casein for 30 minutes at 100° or for 2 hours decreased its growth value no more than did the cold water washing. Likewise, dry heating for these periods and for 8 hours left the protein unchanged from its original value. It must be concluded that heating casein at 100° for 8 hours or less causes no loss of biological value.

It seemed possible that harmful products of the heating might account for the decreased growth of animals fed the more severely heat-treated samples. An experiment was devised to test this supposition. Diets containing 12% raw and washed casein were fed parallel with those containing casein which had been heated for 8 hours at 130° and the same thoroughly washed in cold distilled water. Another group of rats was given raw casein on which had been evaporated the washings from the heated casein. It is obvious that the water-soluble constituents of the heated casein had no injurious effect nor did the washing of the heated protein produce any change in its growth value.

In figure 2 the time and temperature relations of the damage are depicted. Apparently heating for 2 hours at 130° does as

much damage as heating at 120° for 8 hours. Whether heating more than 8 hours at 100° under these conditions would produce significant change can only be conjectured.

On the other hand, it is reasonable to suppose that with increased temperatures, above 140°, heat exposures shorter than 30 minutes may produce damage. Indeed the results of Fairbanks and Mitchell ('35) on milk proteins plainly indicate this likelihood.

The findings of Seegers and Mattill ('35 a, b) on meat proteins indicate increased damage with increasing temperatures

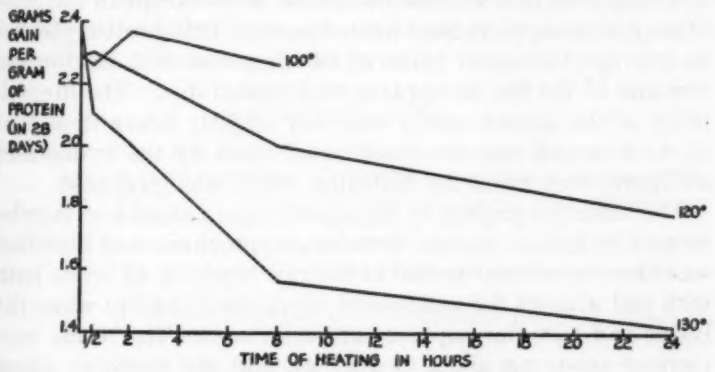

Fig. 2 Decrease in gain in body weight per gram eaten by young rats produced by varying periods of heating casein at 100°, 120° and 130°.

and length of heating. Morgan and Kern ('34) also reported increasing loss of biological value in beef protein with increasing temperature of cooking. Chick, Boas-Fixsen, Hutchinson and Jackson ('35) found much the same to be true of casein, its value being reduced by heating at 150° for 66 hours but not at 112° to 125° for 72 hours. However, Boas-Fixsen and Jackson ('32) in an earlier experiment found that 'light white' casein heated for 3 days at 112° suffered a loss of biological value, declining from 76 to 54.

The explanation of this phenomenon of progressive loss of biological value of proteins with increasing temperature and

duration of heating is not yet at hand. Whether it be due largely to changes in digestibility or to some molecular or intramolecular rearrangement of certain amino acids, the damage is effected gradually and no critical temperature independent of the time element appears to have been established.

SUMMARY

The biological value of casein heated at 140° for 30 minutes alone and with supplements of lysine, cystine and tyrosine was in all cases inferior to that of the corresponding raw protein both with and without the amino acids except in the case of the lysine-supplemented heated casein. This protein yielded an average biological value of 64 compared with 69 for the raw and 57 for the unsupplemented heated diet. The digestibility of the heated casein was only slightly lower than that of the raw and was decreased in all cases by the amino-acid additions even when the biological value was increased.

The value for growth of the heated casein alone and supplemented by lysine, cystine, tyrosine, tryptophane and histidine was likewise inferior to that of the raw casein at all levels both with and without the amino-acid supplements except when the lysine and histidine supplements were used. The lysine supplement made up about two-thirds, and the histidine about one-half of the 14 to 19% loss of value caused by the heat treatment.

Further growth tests upon young rats showed that the decrease in nutritive value caused by heat is proportional to the temperature and time of heating. Cold or hot water washing of raw casein decreased its growth value by 15 to 20% but similar washing of heated casein had no effect nor did the latter washings appear to contain any toxic substances.

Heating casein at 100° up to 8 hours did not decrease its growth value. Heating at 120° up to 2 hours had no effect but heating for 8 hours or more caused increasing amounts of damage. Heating casein at 130° for 30 minutes caused perceptible loss of growth value which loss was progressively increased by lengthening the heating period to 2, 8 and 24 hours.

CONCLUSIONS

It is concluded that lysine is the first amino acid damaged when casein is heated at 140° for 30 minutes, and histidine the second. Histidine damage does not occur at 130°. Cystine, tyrosine and tryptophane are not affected by heating at 140°. The damage is not due to oxidation.

From the digestibility and metabolic data it is concluded that the heat damage to lysine and possibly to histidine and perhaps other amino acids destroys the identity of these amino acids and partly decreases the absorbability of their nitrogen. The nature and extent of such heat damage to a given protein depends not only on the temperature and time of heating but the molecular structure of the protein.

LITERATURE CITED

BOAS-FIXSEN, M. A., J. C. D. HUTCHINSON AND H. M. JACKSON 1934 The biological values of proteins. V. The comparative biological values of the proteins of whole wheat, whole maize and maize gluten, measured by the growth of young rats. Biochem. J., vol. 28, p. 592.

BOAS-FIXSEN, M. A., AND H. M. JACKSON 1932 The biological value of proteins. IV. The biological values of the proteins of wheat, maize and milk. Biochem. J., vol. 26, p. 1919.

CHICK, H. J., M. A. BOAS-FIXSEN, J. C. D. HUTCHINSON AND H. M. JACKSON 1935 The biological value of proteins. VII. The influence of variation in the level of protein in the diet and of heating the protein on its biological value. Biochem. J., vol. 29, p. 1712.

FAIRBANKS, B. W., AND H. H. MITCHELL 1935 The nutritive value of skim milk powders, with special reference to the sensitivity of milk proteins to heat. J. Agri. Res., vol. 51, p. 1107.

GREAVES, E. O., AND A. F. MORGAN 1934 Nutritive value of raw and heated casein with and without added amino-acids. Proc. Soc. Exp. Biol. and Med., vol. 31, p. 506.

KON, S. K., AND Z. MARKUZE 1931 The biological values of the proteins of breads baked from rye and wheat flours alone or combined with yeast or soya bean flour. Biochem. J., vol. 25, p. 1476.

MORGAN, A. F., AND L. E. KEEN 1934 The effect of heat upon the biological value of meat protein. J. Nutrition, vol. 7, p. 367.

MORGAN, A. F., AND F. B. KING 1926 Changes in biological value of cereal proteins due to heat treatment. Proc. Soc. Exp. Biol. and Med., vol. 23, p. 353.

MORGAN, A. F., F. B. KING, R. E. BOYDEN AND V. A. PETRO 1931 The effect of heat upon the biological value of cereal proteins and casein. J. Biol. Chem., vol. 90, p. 771.

OSBORNE, T. B., AND L. B. MENDEL 1916 A quantitative comparison of casein, lactalbumin and edestin for growth or maintenance. J. Biol. Chem., vol. 26, p. 1.

SEEGERS, W. H., AND H. A. MATTILL 1935 a The nutritive value of animal tissues in growth reproduction and lactation. III. The nutritive value of beef heart, kidney, round and liver after heating and after alcohol extraction. J. Nutrition, vol. 10, p. 271.

———— 1935 b The effect of heat and hot alcohol on liver proteins. J. Biol. Chem., vol. 110, p. 531.

THE BIOLOGICAL VALUE OF MILK AND EGG PROTEIN IN YOUNG AND MATURE RATS

EMMA E. SUMNER

Department of Vital Economics, University of Rochester, Rochester, New York

(Received for publication February 25, 1938)

The value of milk or egg protein in human nutrition has been recognized for many years. Mitchell ('24 b), Mitchell and Carman ('24, '26) have shown the superiority of these protein foods for maintenance and slow growth in young rats. Little emphasis has been placed on the fact that the biological value of whole egg has not been surpassed by any other food tested.

Many of the results obtained on rats by the 'balance sheet' method are made difficult of comparison because of the wide fluctuations (3.1 to 12.0%) in the protein level of the experimental diets. Mitchell ('24 b) and Chick et al. ('35) have shown that the percentage of protein in the ration has a profound effect on the resulting biological values.

Another factor which might have a direct bearing on the problem, is the age range of the animals used by different workers. Mitchell ('24 a), however, observed approximately the same biological values for rats of widely different weights. That a difference might exist between young and mature rats was suggested by the work of Osborne and Mendel ('19). They found that the protein of white flour was adequate for maintenance of adult rats, but inadequate for growth of the young. The literature on the biological value of proteins affords few instances in which an opportunity is given to compare results on young and mature rats fed at the same percentage level. For the proteins of corn at a 5% level

in the diet, Boas-Fixsen and Jackson ('32) report a biological value of 84 for adult rats; Mitchell ('24 b) of 72 for young rats. To wheat endosperm at a 7% level, Boas-Fixsen and Jackson assign a value of 61; at an 8% level Mitchell and Carman ('26) of 52. Recently French and Mattill ('35) obtained decidedly lower biological values for white, whole wheat and rye breads in young rats than for the same animals after they had reached maturity. That the age of an animal may influence the biological value of a protein, other experimental conditions being the same, receives support in work completed in this laboratory during 1931 and 1933.

The biological value experiments to be described were carried out on rats primarily for the purpose of securing figures to compare with those for human subjects (Sumner and Murlin, '38). As year-old animals would more nearly approximate the age grouping of the human subjects, the young rats were put back on the regular stock diet [1] after the first series of experiments had been completed, and kept until they reach the desired age.

PLAN OF INVESTIGATION

To assess the value of the proteins of dried whole egg and skim milk powder in the nutrition of rats, two methods were used, 1) 'paired feeding' and 2) the nitrogen 'balance sheet' determination.

The dried whole egg powder was purchased from Jaburg Brothers, New York City. The skim milk powder [2] was obtained locally. The diets were constructed to contain approximately 5 or 8% of crude protein. Only the 8% level was used for the paired-feeding experiments and for the determination of the biological value on young rats. During the standardizing periods, three low nitrogen diets, containing either 0.65, 0.16 or 0.09% of nitrogen were employed. Mitchell and Carman ('26) have shown that the inclusion of from 0.60 to 0.75% of egg nitrogen in the diet does not increase

[1] Purina Chow.
[2] Klim.

the excretion of urinary or fecal nitrogen above that on the nitrogen-free diet, and in addition has the added advantage of being more palatable.

Previous to the preparation of the diets, the egg powder was extracted with cold ethyl ether to remove part of the fat. An amount of lactose equal to that furnished by the milk powder was included in both the egg and the low nitrogen rations. The fat content of the egg and the milk diets was similarly equated. At the 8% level, 11.61 gm. of egg powder provided 0.77 gm. of fat, while 21.89 gm. of skim milk powder

TABLE 1

Composition of diets

	8% level		*5% level*		*Low N*		
	Egg	Milk	Egg	Milk	0.65%	0.16%	0.09%
Ether extracted egg	11.61	....	7.05	....	5.72	1.40	....
Skim milk powder	....	21.89	....	14.01	....	....	....
Hydrogenated cottonseed oil [1]	11.23	11.23	11.58	11.58	11.78	11.95	12.00
Egg fat	....	0.50	....	0.09	....	....	....
Salt mixture [2]	4.50	4.50	4.50	4.50	4.50	4.50	4.50
Lactose	10.60	....	6.80	....	10.60	10.60	10.60
Cellu flour	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Cornstarch	58.06	57.88	66.07	65.82	63.40	67.55	68.90

[1] Crisco.

[2] Hawk and Oser ('31) modification of the Osborne and Mendel salt mixture.

contained 0.27 gm. Hence the difference, 0.5 gm. was added as egg fat to the milk diet. The composition of the diets is given in table 1.

Accurate food consumption records were kept for individual rats in all experiments. The rats were of Wistar strain unless otherwise noted.

Each rat received a daily supplement, fed separately from the rest of the diet, of 2 drops of cod liver oil [3] and 25 mg. of vitamin B complex concentrate.[4] The latter supplied from 1.71 to 2.05 mg. of nitrogen which is included in the total

[3] Patch's.

[4] Harris.

amount ingested per day and hence figures in the calculation of the biological value.

To insure adequate caloric intake, the fuel value of the food eaten was computed and compared with the basal heat production of slow growth rats of different ages reported by Horst, Mendel and Benedict ('34) plus allowance for activity. When the caloric intake fell below the theoretical requirements, the data for that particular period were discarded. With insufficient food, especially during an experimental protein period, Boas-Fixsen ('30) noted a lowering of the biological values. This has been confirmed by Mason and Palmer ('35) who also report that for large adult rats, no effect is observed if the amount of food ingested is low in the nitrogen-free period.

The paired feeding experiment, which continued for 42 consecutive days, followed the technic of Mitchell and Beadles ('30). Young rats varying from 50 to 80 gm. in weight were used, litter mates being paired as to weight and sex. Food intakes were controlled so that the two rats of each pair received the same quantity of food. The rat consuming the least food, which was always the animal on the milk ration, determined the amount that its pair-mate received. For the determination of the biological value, Mitchell's ('24 a) method was adopted. The rats were of two age groups; young animals about 45 days old and year-old animals, some of which had previously been used at the younger age.

For separation of urine and feces, metabolism cages similar to those described by Ackroyd and Hopkins ('16) were employed. Water was supplied in an inverted tube, in place of the open container used by the former investigators. In addition to the cone-shaped sleeves, a small crescent-shaped piece of tin was soldered across the bottom of the arm opening. With this arrangement, there was practically no scattering of food.

Before the start of each collection period, the galvanized wire mesh bottom, large funnel and separating bulb were thoroughly cleaned and finally rinsed with warm 0.5% H_2SO_4.

The cylinder for the collection of the urine contained 5 cc. of 0.5% H_2SO_4 and 2 drops of 10% thymol in toluene. Urine and feces were collected daily. At the time of collection, the cage bottom, funnel and separating bulb were washed with warm 0.5% H_2SO_4 with the help of a rubber 'policeman.' During the cleaning process, the rat was kept in a small wire cage which rested in a large evaporating dish. Thus any urine or feces passed could be collected quantitatively. All cage washings were filtered and added to the urine. If any of the feces became contaminated by urine, it was well rinsed with warm acid and the rinsings added to the urine flask. Urine and cage washings were pooled for the duration of each collection period, then made up to a convenient volume and aliquots taken for nitrogen determination.

To test for loss of urinary nitrogen over a 24-hour period, 10 cc. of urine of known nitrogen content were trickled from a burette over the cage bottom at intervals during the day, and allowed to stand over night. The metabolism cage had previously been prepared as if for an actual collection period. The next morning cage, funnel and separating bulb were washed as previously described. In one instance, nitrogen recovery was 100% and in the other 97%.

Feces were collected daily in a Kjeldahl flask containing concentrated H_2SO_4. The entire amount was digested at the end of the period, transferred quantitatively to a volumetric flask, cooled, made up to volume and aliquots taken for determination of nitrogen.

All nitrogen determinations were made by the macro-Kjeldahl method in triplicate on the diets and in duplicate on urine or the fecal digest. The lactose content of milk powder was determined by the Bertrand method described by Woodman in 'Food Analysis' second edition, page 117; fat, by the Werner-Schmidt method as given in Leach 'Food Inspection and Analysis' fourth edition, page 126. Egg powder was extracted with cold ethyl ether in the Soxhlet extractors for 16 hours and the amount of ether-extracted substance determined by weighing.

EXPERIMENTAL RESULTS

Paired feeding experiments. Of the twenty-two young animals used in the paired feeding experiment, the rat receiving the egg diet made in every instance the greater gain in total weight. The data for the five pairs selected as being typical of the entire group of eleven pairs, are given in table 2.

If an event may occur with equal probability in either of two ways, that is, either the egg-fed or the milk-fed rat could show the greater gain, then in a random selection of eleven trials the frequency of its occurrence in one way is given by the expansion of the binomial distribution $(\frac{1}{2}+\frac{1}{2})^{11}$. Its occurrence eleven times in either way would result from chance only once in 1024 observations.

TABLE 2

Growth value of milk and egg protein (all weights in grams)

	Pair 1		*Pair 2*		*Pair 3*		*Pair 4*		*Pair 5*	
	Egg	Milk	Egg	Milk	Egg	Milk	Egg	Milk	Egg	Milk
Grams protein eaten	20.0	20.3	14.8	15.1	24.1	24.6	25.8	26.4	24.4	24.9
Initial weight	58.0	57.0	62.0	62.0	70.0	70.0	61.0	62.0	64.0	65.0
Final weight	116.0	92.0	98.0	91.0	156.0	136.0	130.0	113.0	128.0	109.0
Gain	58.0	35.0	36.0	29.0	86.0	66.0	69.0	51.0	64.0	44.0

Nitrogen 'balance sheet' determinations. a. Experiments on young rats. Two experiments were carried out using young rats ranging in weight from 65 to 100 gm., and in age from 40 to 50 days. The first group consisted of six animals. The low protein ration containing 0.65% nitrogen was fed for a period of 6 days. Urine and feces were collected during the last 3. Half of the rats received the milk diet and the other half the egg diet for 18 consecutive days. Urine and feces were collected the last 14 days in two periods of 7 days each. The average of five acceptable periods gives a biological value of 80 (77 to 86) for milk; and of six periods on egg, 99 (97 to 100).

b. Experiments on year-old rats. Thirty mature rats, approximately 1 year old and ranging in weight from 200 to 360 gm., were employed for the balance of the work, which consisted of experiments 3, 4 and 5.

Experiment 3. All of the ten animals used in this experiment had previously served as subjects at the younger age. The 0.16% low nitrogen diet was fed during the standardizing periods. The egg and the milk diets were tested at both the 5 and 8% level, the experiments at the 5% level being carried out immediately after the first low nitrogen period. Another standardizing period intervened between the two levels of protein feeding. Half of the rats received the milk ration in the first period; the other half, egg. For six of the total of ten rats, the proteins were reversed in consecutive periods; for the other four rats, two periods were run on the same protein. All feeding periods were of 8 days duration, urine and feces being collected for the last 4 days. At the 8% level an average of nine acceptable periods gives a biological value for egg of 77 (71 to 96); of twelve periods for milk an average of 65 (56 to 69). Higher values were obtained for both proteins at the 5% level. The average for seven periods for egg is 94 (82 to 100); for milk, an average of nine periods is 78 (64 to 100).

Experiment 4. To increase the number of cases in the mature group at 8% level, rats were purchased from the Albino Supply Company which were from 11 to 11½ months old and ranged in weight from 295 to 325 gm. The six largest animals were selected for the experiment. The standardizing period, during which the diet supplying 0.09% nitrogen was fed, lasted 8 days. The protein periods were of 9 days duration, the two proteins being reversed in consecutive periods on the same rat. Half the animals started on the egg, the other half on the skim milk ration. Urine and feces were collected for the last 4 days of the low nitrogen periods and for the last 5 days of the protein regime. The same low range is obtained for the biological value of milk powder as in the first group of rats, the average for the six periods being 62 (47 to 79). Three of the rats gave values of 49, 50 and 50. As the food consumption was adequate, the results were included in the average. These low values were due to an enormous increase in urinary nitrogen during the milk period in comparison with that of the preceding egg period. In contrast with the continued low values for milk, those for egg are as high as any observed for the young rats, the six periods averaging 97 (93 to 100). For all adult rats in experiments 3 and 4, fed at an 8% level, the biological value of eighteen periods on milk averages 64 (47 to 79), and fifteen periods on egg 85 (71 to 100).

Experiment 5 Experiment 5 was undertaken to round out the number of observations on year-old rats at the 5% level. Two groups, totalling fifteen animals were used. Both groups received the diet containing 0.09% nitrogen for the standardization period. All feeding periods were of 8 days duration, urine and feces being collected for the last 4. An average of twelve periods gives a biological value for milk of 80 (56 to 91) and for eggs of 93 (63 to 100). A grand average of all values at the 5% level assigns milk a rating of 78 (56 to 100) for twenty-two periods, and egg of 94 (63 to 100) for twenty periods.

All the work on rats had been completed before Schneider ('35) described his chromic oxide and ferric oxide method of

TABLE 3

Biological value of milk and egg protein at two intake levels in rats at different ages

			Biological value of milk			Biological value of egg		
Age	*Weight,*	*Protein level*	Number of periods	Average,	Range,	Number of periods	Average,	Range,
	gm.	%		%	%		%	%
50 days	65–100	8	14	84	77–92	20	97	88–100
1 year	200–300	8	18	64	47–79	15	85	71–100
1 year	260–365	5	22	78	56–100	20	94	63–100

marking feces for short collection periods, or before Ashworth ('35) had shown that certain proteins may influence the endogenous nitrogen excretion. As the same protein was fed throughout the period between standardizations in the study on young rats, the influence of a nitrogenous food, other than the one under consideration was avoided.

DISCUSSION OF RESULTS

The average results of these experiments on two age groups of rats are summarized in table 3.

For young rats fed at an 8% level, Mitchell has reported a value of 85 for milk and 94 for egg. The averages 84 and 97 from the present study are in good agreement with these figures. No value has been found in the literature for the evaluation of whole egg protein for maintenance of adult rats. Chick et al. ('35) gave a biological value of 86 for milk powder

at a 7% level for maintenance of mature rats. This is considerably higher than the values observed in this study for year-old rats either at the 8 or the 5% protein level, 64 and 78, respectively.

With year-old rats, the biological value of milk and of egg at the 8% level is definitely lower than for the young animals. This is true not only for the average values, but for each rat which participated in the two age groups studies and received the same protein ration in each experiment.

Individual values are given in table 4.

Apparently with proteins of good quality such as milk or egg, less nitrogen is needed to cover the maintenance requirement of mature rats, than for growth and maintenance of the

TABLE 4

Biological value of milk and of egg protein in the same rat at different ages

Protein	*Rat no.*	*Biological value* 50 days, %	Year-old, %
8% milk	7	84[1]	69[1]
	9	83[1]	66[1]
	10	77	63
8% egg	12	100[1]	95[1]
	14	93[1]	85[1]
	17	100[1]	51

[1] Average of two acceptable periods.

young. The results are not so clear cut for egg as for milk, although for the former there is a difference of 12 between the average values for the two age groups. Therefore these experiments lend support to the conclusions of French and Mattill ('35) that the protein requirement of young and mature rats may not be the same.

The biological value of both milk and egg protein for adult rats is higher at the 5 than at the 8% level of feeding; 78 as compared with 64 for milk, and 94 with 85 for egg. The higher value for milk at the lower level in the diet confirms Mitchell's findings with young rats, rather than the observation of Chick et al., that there is practically no difference in

the biological value obtained for adult rats at a 7% (86) or at a 5% (89) level in the diet.

From the data of these experiments, both the apparent and the true coefficients of digestibility may be calculated. The average results for both age groups are summarized in table 5.

There is no difference between the absorption of milk or egg protein at the 8% level in young or adult rats expressed either as the apparent or as the true coefficient of digestibility. In adult rats, the apparent coefficients are slightly lower at the 5 than at the 8% level. When expressed in terms of true digestibility the averages fall within the range obtained for both young and old rats at the 8% level.

TABLE 5

Coefficients of digestibility for milk and egg powder

	Level in diet, %	*Number of periods*	*Apparent*		*True*	
			Average, %	Range, %	Average, %	Range, %
Milk						
Age	8	14	79	74–86	95	89–100
Young	8	19	80	77–83	95	88–100
Adult	5	21	71	65–77	98	94–100
Egg						
Young	8	20	81	76–89	95	90–100
Adult	8	17	78	73–83	92	84–100
Adult	5	21	73	50–85	97	76–100

CONCLUSIONS

The biological value of whole egg protein is higher than that of whole milk, whether determined by the paired feeding or by the balance sheet method on young or adult rats.

Feeding at an 8% level in the diet, the biological value is higher for both egg and milk in young rats than in the year-old animals.

In adult rats the biological value for egg and milk proteins is higher at the 5% than at the 8% level of protein in the diet.

These facts lead to the conclusion that the protein requirement for growth in young rats and for maintenance in adult rats is not the same. Less protein of good quality is required for the maintenance of mature rats, than for growth in the young.

The proteins of dried milk or dried egg are equally digestible, whether compared on the basis of the apparent or of the true coefficient of digestibility.

LITERATURE CITED

ACKROYD, H., AND F. G. HOPKINS 1916 Feeding experiments with deficiencies in the amino-acid supply: Arginine and histidine as possible precursors of purines. Biochem. J., vol. 10, p. 551.

ASHWORTH, U. S. 1935 Growth and development with special reference to domestic anmals. XXXVII. Interrelations between protein intake, endogenous nitrogen exeretion and biological value of protein. Mo. Agri. Exp. Sta. Res., Bull., no. 228.

BOAS-FIXSEN, M. A. B. 1930 II. The biological value of purified caseinogen and the influence of vitamin B_1 upon biological values, determined by the balance sheet method. Biochem. J., vol. 24, p. 1794.

BOAS-FIXSEN, M. A. B., AND H. M. JACKSON 1932 IV. The biological values of the proteins of wheat, maize and milk. Biochem. J., vol. 26, p. 1932.

CHICK, H., M. A. B. BOAS-FIXSEN, J. C. D. HUTCHINSON AND H. M. JACKSON 1935 VII. The influence of variation in the level of protein in the diet and of heating the protein on its biological value. Biochem. J., vol. 29, p. 1702.

FRENCH, R. B., AND H. A. MATTILL 1935 The biological value of the proteins of white, wheat and rye breads. Cereal Chemistry, vol. 12, p. 365.

HAWK, P. B., AND B. L. OSER 1931 A modification of the Osborne-Mendel salt mixture. Science, vol. 74, p. 369.

HORST, K., L. B. MENDEL AND F. G. BENEDICT 1934 The influence of previous diet, growth and age upon the basal metabolism of the rat. J. Nutrition, vol. 8, p. 139.

MASON, I. D., AND L. S. PALMER 1935 Utilization of gelatin, casein and zein by adult rats. J. Nutrition, vol. 9, p. 489.

MITCHELL, H. H. 1924 a A method of determining the biological value of protein. J. Biol. Chem., vol. 58, p. 873.

———— 1924 b The biological value of proteins at different levels of intake. J. Biol. Chem., vol. 58, p. 905.

MITCHELL, H. H., AND G. G. CARMAN 1924 The biological value for maintenance and growth of the proteins of whole wheat, egg and pork. J. Biol. Chem., vol. 60, p. 613.

———— 1926 The biological values of the nitrogen of mixtures of patent white flour and animal foods. J. Biol. Chem., vol. 68, p. 183.

MITCHELL, H. H., AND J. R. BEADLES 1930 The paired-feeding method in nutrition experiments and its application to the problem of cystine deficiencies in food proteins. J. Nutrition, vol. 2, p. 225.

OSBORNE, T. B., AND L. B. MENDEL 1919 The nutritive value of the wheat kernel and its milling products. J. Biol. Chem., vol. 37, p. 557.

SCHNEIDER, B. N. 1935 The subdivision of the metabolic nitrogen in the feces of the rat, swine and man. J. Biol. Chem., vol. 109, p. 249.

SUMNER, EMMA E., AND JOHN R. MURLIN 1938 The biological value of milk and egg protein in human subjects. J. Nutrition, vol. 16, pp. 141–152.

THE BIOLOGICAL VALUE OF MILK AND EGG PROTEIN IN HUMAN SUBJECTS

EMMA E. SUMNER AND J. R. MURLIN

Department of Vital Economics, University of Rochester, Rochester, New York

(Received for publication February 25, 1938)

In most of the work on the evaluation of protein foods in human nutrition, a comparison is made between the gross output of nitrogen in urine and feces with the amount ingested, the efficiency of the food depending on the quantity that must be fed in order to bring about nitrogen equilibrium or retention. For more exact determinations this procedure has been modified, so that an estimation is first made of the so-called 'metabolic' nitrogen of urine and feces, thus permitting a comparison between the food nitrogen actually absorbed from the intestine of the subject, and the percentage of this amount retained in the body. Using this latter method the biological value of a protein is obtained, which according to Thomas ('09) is the number of parts of body nitrogen replaced by 100 parts of food nitrogen.

Two widely divergent numerical values have been given for the worth of milk in adult human nutrition. To modified cow's milk, (a preparation consisting of fresh cream with the addition of water, glucose and lactose in amounts to give a composition similar to human milk) Thomas gave a biological value of 100. Martin and Robison ('22) reported a value of 51 for whole milk. This figure seems surprisingly low in contrast with that of Thomas. It must be remembered that the one milk period in the series of experiments undertaken by Thomas lasted only 2 days with considerable variation in nitrogen intake, while the periods of Martin and Robison

were never shorter than 4 days and often continued from 6 to 9 days with constant nitrogen intake. Wagner ('23), using as subjects a group of convalescent children from 10 to 12 years old, most of whom were described as having latent hilus gland tuberculosis, gives a biological value of 60 for milk and 71 for whole egg. Although both these figures were calculated according to the B formula of Thomas, they are not strictly comparable, as the correction for extractive nitrogen was not made for egg as had been done in the case of milk. No biological value for whole egg using human adults as subjects could be found in the literature.

PLAN OF INVESTIGATION

Attempts to ascertain the biological value of powdered whole milk[1] and whole egg flake in human nutrition were undertaken on two different occasions. Estimation of the 'wear and tear' level of nitrogen metabolism involves the ingestion over a period of days of a diet free from protein. Realizing the unpalatability of the low nitrogen diet of Thomas, which was composed entirely of starch, sucrose and lactose, the basal diet of the present study was patterned after than used by Smith ('26) in his work on the determination of the minimum nitrogen excretion. It therefore included in addition to these carbohydrates, small servings of fresh or canned fruits, which were of low nitrogen content. Even with these additions, certain individuals are apparently unable to continue for any length of time on a diet supplying as much as 75% of its calories from such carbohydrate materials as cornstarch, sucrose, lactose and corn syrup. From a total of ten participants only four subjects completed the study. The others, with the exception of one subject who dropped out on account of an abscessed tooth, lasted from 1 to 7 days on the basal diet, before becoming so violently distressed by utter lack of interest in food of any kind, nausea and vomiting, that they had to discontinue the experiment.

[1] Klim.

For several days prior to the diet, subjects Y and B, the survivors of the first group, estimated the time spent at different activities and calculated that an intake of 40 calories per kilogram would amply cover their energy needs. The caloric distribution of their diet was as follows: protein 4%, fat 31% and carbohydrate 65%.

For the second group, represented by subjects X and F, an intake of 50 calories per kilogram was arbitrarily selected. Vitamin B complex concentrate was given from the start of the diet. Notwithstanding, X and F had greater difficulty in the quantitative ingestion of all the carbohydrate food

TABLE 1

Daily food intake. Subject F

Food	*Amount, gm.*	*Food*	*Amount, gm.*
Cornstarch	175	Canned pineapple pulp	50
Hydrogenated cottonseed oil	20	juice	20
Sucrose	134	Lettuce	40
Corn syrup	201	Salad oil	20
Butterfat	40	Apple	100
Grapefruit pulp	80	Adex tablet	1
juice	20	Vitavin B complex concentrate	1 tablet
Lemon juice	40	Milk powder	94
Lactose	36	Egg flake	53

Nitrogen of basal diet 0.55 gm.
Total nitrogen supplied by diet 4.3 gm.
Calories 3300

materials than had Y and B, whose diets lacked the added vitamin. Of the total calories supplied by the diets of X and F, approximately 3% came from protein, 24 to 29% from fat, and 68 to 72% from carbohydrate.

The total food intake per day given in table 1 for subject F is practically identical with that of the other three subjects. The basal diet, which includes all foods except milk and egg, contributed from 0.55 to 0.63 gm. of nitrogen, varying slightly for each individual. With the addition of milk or egg to the basal diet, the nitrogen intake increased to 3.5 to 5.4 gm. for the various subjects.

The division of the food materials into meals was as follows:

Breakfast

Grapefruit and sugar
Cornstarch bread, butterfat and corn syrup
Lactose lemonade

Lunch

Cornstarch bread, butterfat and corn syrup
Canned pineapple
Lactose lemonade
Milk or plain egg omelet

Supper

Cornstarch bread, butterfat and corn syrup
Lactose lemonade
Head lettuce, oil and lemon juice
Raw peeled apple
Milk or plain egg omelet

Whole milk powder [2] was obtained locally. The dried whole egg flake was purchased from H. J. Keith Company of New York City. Butter was brought in from a farm the day it was churned and butterfat prepared by melting over hot water, decanting and filtering through a Koch hot water funnel. After the fat had solidified, salt was worked through it. To increase the palatability of the cornstarch, it was made up into a bread. The batter for this bread was made up separately for each individual according to the following proportions:

100 to 150 gm. cornstarch
8 gm. gum acacia
20 gm. hydrogenated cottonseed oil [3]
5 gm. sucrose
14 gm. corn syrup
1.5 gm. salt

Add gum acacia to cornstarch, and mix thoroughly. To the hydrogenated cottonseed oil, sucrose, corn syrup and salt, add about 1 cup of hot water, and stir until the oil is melted. Add cornstarch and gum acacia and blend thoroughly. It may be necessary to add more water, as the batter should be thin enough to pour readily. Put approximately one-third of this mixture in a well greased $2\frac{1}{2} \times 5$ inch tin. Add $\frac{1}{4}$ teaspoon of any alum baking powder directly to the batter in the pan, stirring until mixed. Bake $\frac{3}{4}$ hour in a slow oven, and then 15 minutes in a moderate oven. Serve while hot.

[2] See footnote 1.
[3] Crisco.

Such articles of the diet as cornstarch, lactose, vitamin B complex concentrate[4] tablets, gum acacia, hydrogenated cottonseed oil, salad oil, corn syrup,[5] canned pineapple, sucrose, apples, grapefruit and lemons were ordered in amounts to last through the experiment. Fresh lots of lettuce were ordered every 3 or 4 days. Samples from a number of cans of each food were pooled at different times and analyses run on these composites. Grapefruit pulp was prepared by removing any adhering membrane or skin, the lemon juice was strained and the apples peeled before serving or sampling. By using a cork-borer, samples for analyses were obtained from several parts of a dozen heads of lettuce, or of apples, and analyses run on the mixed samples of each food.

Triplicate determinations for nitrogen were carried out on all the foods by the macro-Kjeldahl method. For the second group of subjects, one-tenth of each dietary constituent was pooled and the total nitrogen determined for the mixture. The feces of the different periods were separated by the alternate use of carmine and charcoal. Urine was collected daily from 8.00 A.M. to 8.00 A.M. Toluene was used as the preservative. For subjects Y and B the feces were weighed, mixed and aliquots taken for analysis as soon as passed. Nitrogen determinations were run in triplicate on the fresh feces, and in duplicate on the 24-hour collection of urine. For subjects X and F, one-tenth of each stool was pooled, digested in 20% sulfuric acid, transferred to a volumetric flask, cooled, made up to volume and duplicate samples taken for nitrogen determination.

The duration of the diet periods varied somewhat for the different subjects.

Subjects Y and B

Period 1. Low nitrogen. Both subjects ate the basal ration for 1 preliminary day. Then followed a collection period of 7 days. The sum of the average nitrogen excreted in the

[4] Harris.
[5] Karo.

urine and feces during the last 3 days is taken as the amount to be supplied by the added protein, either in the form of milk powder or as egg flake.

Period 2. Milk was added to the basal ration over a period of 4 days. Urine and feces were collected for the entire period, but only the average of the last 2 days is used in the calculations.

Period 3. Egg flake. Subject B followed the milk diet with a 3-day period, in which an equal amount of nitrogen was supplied by the egg flake. Collections were made during the 3 days. The data obtained for the last 2 days are used in calculating the biological value.

Period 4. Low nitrogen. For subject Y, the second low nitrogen period immediately followed the milk period, while for subject B, the egg flake period preceded it. For both subjects the duration of the diet was 3 days. Urine and feces were collected for the period. An average between the last 3 days of the first low nitrogen period, and the last 2 days of the second, is taken as representing the minimum nitrogen excretion, and is used in computing the biological value of the nitrogen supplied by milk or egg.

Subjects X and F

Period 1. Low nitrogen. The basal diet was eaten for one preliminary day before starting the collection of urine and feces, which lasted for the next 6 days. The sum of the average nitrogen excreted in the urine and feces during the last 3 days of the period is taken as representing the amount of nitrogen to be furnished by the added proteins.

Period 2. Egg flake as the source of protein. Urine and feces were collected for the 7-day period, the average nitrogen excretion of the last 3 days being used for the computations.

Period 3. Milk powder supplied an amount of nitrogen equal to that received from the egg flake, over a 6-day period. Urine and feces were collected daily. The data for the last 3 days of the diet are used in making a comparison with the egg period.

EXPERIMENTAL RESULTS

The daily nitrogen intake and the average output in urine and feces is given for the four subjects in table 2.

For the calculation of the biological value of milk and egg protein, the method developed by Mitchell ('24) was used. In the case of human subjects, the assumption was made that the nitrogen excreted in the urine and feces during the low nitrogen diet was characteristic of each individual. No attempt was made to correlate fecal nitrogen with food intake

TABLE 2

Nitrogen balance data—biological value study

	N daily intake, gm.	*Daily N output* Urine, gm.	Feces, gm.	Total, gm.	*N balance, gm.*	*N average urine or feces*
Subject Y [1]						
Low N 1	0.58	2.31	0.65	2.96	—2.38	Last 3 of 7 days
Milk period	3.55	3.50	0.41	3.91	—0.36	Last 2 of 4 days
Low N 2	0.58	2.27	0.74	3.01	—2.43	Last 2 of 3 days
Average low N	0.58	2.29	0.69	2.99	—2.41	
Subject B						
Low N 1	0.63	3.12	0.93	4.05	—3.42	Last 3 of 7 days
Milk period	3.78	4.78	0.99	5.77	—1.99	Last 2 of 4 days
Egg period	3.78	4.55	0.64	5.19	—1.41	Last 2 of 3 days
Low N 2	0.63	2.89	0.99	3.88	—3.25	Last 2 of 3 days
Average low N	0.63	3.00	0.96	3.96	—3.33	
Subject X						
Low N	0.55	3.75	0.27	4.02	—3.47	Last 3 of 7 days
Egg period	5.39	5.54	1.02	6.56	—1.17	Last 3 of 7 days
Milk period	5.36	5.52	1.63	7.15	—1.79	Last 3 of 6 days
Subject F						
Low N	0.55	2.77	0.56	3.33	—2.78	Last 3 of 7 days
Egg period	4.38	3.77	1.01	4.78	—0.40	Last 3 of 7 days
Milk period	4.32	3.72	1.21	4.93	—0.61	Last 3 of 6 days

[1] Woman.

as dry matter had not been determined in the diet, or urinary nitrogen to body weight as had been done for the rats (Sumner, '38).

The biological value of a protein can be expressed as

$$\frac{\text{N retained in the body}}{\text{Food N absorbed}} \times 100$$

In the expanded formula this becomes,

$$\text{B.V.} = 100 \times \frac{[\text{Intake (P)} - (\text{F.N. (P)} - \text{F.N. (M)}) - (\text{U.N. (P)} - \text{U.N. (M)})]}{\text{Intake (P)} - (\text{F.N. (P)} - \text{F.N. (M)})}$$

where F signifies feces; U, urine; N, nitrogen; P, the experiment with the protein under investigation; M, the low nitrogen experiment.

Table 3 gives the biological values computed according to Mitchell and by the two formulae of Martin and Robison.

For egg flake the average biological value is 65, for milk powder 62, showing an insignificant difference in the retention of absorbed nitrogen for the two proteins. The individual differences for subjects B and X are slightly larger than that shown by the average. Compared on the basis of nitrogen balance, the egg periods are less negative than those of

TABLE 3

Biological value of milk and egg calculated according to

Subject	*Mitchell*		*Martin and Robison*			
	Egg flake, %	Milk powder, %	Egg flake, %		Milk powder, %	
			A	B	A	B
Y[1]		65			59	62
B	59	53	51	55	47	44
X	61	56	63	56	63	49
F	74	73	74	70	75	70
Average	65	62	63	60	61	56

[1] Woman subject

milk, although for subject F this difference is less pronounced than for the others. The explanation lies in a somewhat greater excretion of fecal nitrogen during the milk period for Y, B and X.

From the experimental data both apparent and true coefficients of digestibility may be calculated. The averages for egg flake are 80 and 92, respectively, for apparent and true digestibility; for milk, 76 and 90. The apparent absorption of dry milk is somewhat lower than that of egg flake. If comparison is made after allowing for metabolic fecal nitrogen, the difference is very small. About the same degree of

improved assimilation is shown by both proteins, when the true is compared with the apparent coefficient of digestibility.

DISCUSSION OF RESULTS

Martin and Robison ('22) assigned a biological value of 51 to the proteins of cow's milk.

It is interesting to note that the same low range values were arrived at in this experiment, in contrast to the high valuation of 100 given by Thomas ('09).

The relative rating of the two proteins is the same for human subjects and for rats, although the superiority of egg is less pronounced for the former. From table 4 it may be seen that by comparing human subjects at a 3 to 4% level

TABLE 4

Comparison of biological value in human subjects and in rats

			Biological value milk		*Biological value egg*	
Subject	*Age*	*Protein calories, %*	Number of cases	Average, %	Number of cases	Average, %
Human	23–24 years	3.0–4.0	4	62	3	65
Rats	11–12 months	5.0	22	80	20	94
	11–12 months	8.0	18	64	15	85
	50 days	8.0	14	84	20	97

with adult rats at 8% level almost identical values for milk powder are observed in the two species. This is not the case for egg protein. Rather the 8% level for rats gives a B.V. 20% above that at 3 to 4% for the human. At the 5% level for the rat the B.V. of both egg protein and milk protein is much greater than at the 8% level. For a number of human subjects in the egg-replacement study, described in another paper (Sumner, Pierce and Murlin, '38), nitrogen equilibrium was practically attained by several members of the diet squad during the second egg period. Hence, there is the possibility that had the duration of the human biological value experiment been longer, a higher percentage retention of egg nitrogen might have been noted. As the young men who served

as subjects were fully grown and the food supplied an amount of nitrogen equal to that lost from the body during a low protein feeding period, the assumption is made that the resulting biological values were those of maintenance. For the adult rats, however, the maintenance value would be more nearly approximated at the 5% than at the 8% level; that even higher results for maintenance may be obtained for mature rats at still lower levels in the diet has been demonstrated by Chick et al. ('35) who report a value of 89 for milk at a 3.4% level. Therefore, it would seem that the actual biological value for maintenance in the two species may differ, the proteins of milk and egg being utilized with greater efficiency by the rat. In fact, the work of Mitchell and Hamilton ('31) suggests that there may be a species difference for certain proteins. Both the pig and the rat made use of the proteins of corn with equal efficiency, but the proteins of linseed and cottonseed meal were not nearly so well utilized by the pig.

Adolph ('36) in a lecture given at the University of Rochester, told of the onset of nutritional edema, characterized by a low serum albumin, in those Chinese who were compelled to subsist on millet and corn in times of famine. Yet exceptionally high biological values have been reported for these cereals by investigators using rats as the experimental animal. According to Niyogi et al. ('34) millet rates 90, while Chick et al. ('35) assign to maize endosperm a value of 88.

In their work on bread, French and Mattill ('35) call attention to the close agreement between the values of 83 and 81 obtained on human subjects by the replacement method using milk as a standard, and on mature rats by the biological procedure. In contrasting results from two experiments employing different procedures, it must be remembered that a biological value expresses the ability of a protein to replace body nitrogen, while a replacement value measures to what extent one protein can replace another. Thus, for a truly valid comparison, it is necessary to assume that the standard

protein of the replacement method is able to replace body nitrogen gram for gram. That neither milk nor egg, the two proteins most commonly used as standards, is capable of this in human nutrition is strongly indicated by the results of Wagner, Martin and Robison, and the present research. The only dissenting value reported is that of Thomas ('09), who assigned to a milk preparation a value of 100, an excellence which other investigators concede to be too high.

The proteins of milk and egg seem to be equally digestible compared one with the other, whether the results are obtained on human subjects or rats. For milk powder an apparent coefficient of 76 was found for the human subjects, and 75 for the rats; for egg 80 and 76, respectively. Expressed in terms of true digestibility, milk powder averaged 90 for humans and 94 for rats; egg flake 92 and 92, respectively.

CONCLUSIONS

The superiority of egg over milk is less pronounced in the nutrition of adult human subjects than in young or mature rats.

Under the conditions of this experiment, the biological value of milk or egg protein for maintenance in adult human subjects, is lower than that observed for maintenance in adult rats, notwithstanding that it was fed to human subjects at a lower level. The observations on human subjects are too few in number to state definitely whether this is actually a species difference.

The proteins of milk and egg are equally well utilized by human subjects or by rats, whether measured by the apparent or true coefficient of digestibility.

Egg protein is only slightly superior to milk in replacing nitrogen at the 3 to 4% level in human subjects. This apparent equality of the proteins may be due to the limited number of observations. The value for milk agrees with that obtained by Martin and Robison ('22) for a very similar method.

LITERATURE CITED

ADOLPH, W. H. 1936 Lecture given at the University of Rochester.

CHICK, H., M. A. B. BOAS-FIXSEN, J. C. D. HUTCHINSON AND H. M. JACKSON 1935 VII. The influence of variation in the level of protein in the diet and of heating the protein on its biological value. Biochem. J., vol. 29, p. 1702.

FRENCH, R. B., AND H. A. MATTILL 1935 The biological value of the proteins of white, wheat and rye breads. Cereal Chemistry, vol. 12, p. 365.

MARTIN, C. J., AND R. ROBISON 1922 The minimum nitrogen expenditure of man and the biological value of various proteins for human nutrition. Biochem. J., vol. 26, p. 407.

MITCHELL, H. H. 1924 A method of determining the biological value of protein. J. Biol. Chem., vol. 58, p. 873.

MITCHELL, H. H., AND T. S. HAMILTON 1931 The nutritive value for growing swine of the proteins of linseed meal and of cottonseed meal, both alone and in combination with the proteins of corn. J. Agri. Research, vol. 43, p. 743.

NIYOGI, S. P., N. NARAYANA AND B. G. DESAI 1934 The nutritive value of Ragi (eleusine coracana). Ind. J. Med. Res., vol. 22, p. 373.

SMITH, M. 1926 Minimum endogenous nitrogen metabolism. J. Biol. Chem., vol. 68, p. 15.

SUMNER, EMMA E., H. B. PIERCE AND J. R. MURLIN 1938 The egg-replacement value of several proteins in human nutrition. J. Nutrition, vol. 16, p. 37.

THOMAS, K. 1909 Über die biologische wertigkeit der Stickstoffsubstanzen in verschiedenen Nahrungsmitteln. Arch. anat. u. Physiol., Bd. 219.

WAGNER, R. 1923 Zur biologischen Wertigkeit der stickstoffhaltigen Nahrungsmittel. Ein Beitrag zum Minimum gesetz in der Ernährungslehre (Nach Versuchen am wachsenden Organismus). Zeitschr. f. die ges. exp. Med., Bd. 33–34, S. 250.

THE EFFECT OF VARIOUS FRACTIONS OF LIVER ON EXPERIMENTAL CANINE BLACKTONGUE [1,2]

HAROLD I. HARVEY,[3] DAVID T. SMITH, ELBERT L. PERSONS AND MARGARET V. BURNS

Department of Medicine, Duke University

FOUR FIGURES

(Received for publication January 26, 1938)

In September, 1937, Elvehjem, Madden, Strong and Woolley reported the isolation of nicotinic acid amide from liver and showed that nicotinic acid (Eastman) would cure experimental blacktongue. A complete report of their studies appeared in March, 1938. Dann ('37), Street and Cowgill ('37), Dann and SubbaRow ('38), Smith, Margolis and Margolis ('38) have confirmed Elvehjem's results on blacktongue. The last-mentioned authors found that nicotinic acid was still effective after being autoclaved at 15 pounds pressure for 5 hours.

Nicotinic acid has been used successfully in the treatment of human pellagra by Fouts, Helmer, Lepkovsky and Jukes ('37), Smith, Ruffin and Smith ('37), Harris and Hassan ('37), Spies, Cooper and Blankenhorn ('37, '38). These

[1] This is the eleventh in a series of coordinated studies on pellagra in man and associated deficiency diseases of animals from the Departments of Physiology, Pathology, Bacteriology and Medicine of Duke University with the cooperation of Dr. Y. SubbaRow of the Department of Biochemistry of Harvard Medical School. Previous publications are listed in the 'Literature Cited' as Smith ('32), Ruffin and Smith ('34), Smith and Sprunt ('35), Dann ('36), Ruffin and Smith ('37), Smith and Ruffin ('37), Smith, Persons and Harvey ('37), Smith, Ruffin and Smith ('37), Dann ('37) and Smith ('38).

[2] The expenses of this work were covered in part by a grant from the Lederle Biological Laboratories, Pearl River, New York.

[3] Aided by the Henry Strong Denison Medical Foundation.

studies were all made independently and almost simultaneously.

The present study, which has been in progress for over 4 years, was planned to devise a method for testing in dogs, the blacktongue curative value of various substances and to assay certain liver extracts and fractions of liver extract which have been used under controlled conditions, in the treatment of human pellagra. The standard curves obtained with liver may be used in assaying the potency of nicotinic acid. The immediate results with nicotinic acid seem quite as good as those obtained with liver, but it has not yet been demonstrated that the prolonged administration of this substance will be equally effective.

METHOD

Adult dogs of mixed breeds and both sexes were used. For convenience in handling, animals weighing between 10 and 15 kg. were used. Undernourished dogs and those with mange or intestinal parasites were used only after treatment and reconditioning on an adequate stock diet.

Goldberger's diet no. 123 was modified by substituting the Underhill and Mendel ('28) more complete salt mixture for the sodium chloride and calcium carbonate, yellow cornmeal for the white cornmeal, and by doubling the amount of cod liver oil. Commercial casein (Merck) was leached with acidulated water and extracted with 95% alcohol.[4] The diet used consisted of yellow cornmeal, 400 gm.; coarsely ground California black-eyed peas (Vigna sinensis), 50 gm.; casein, 60 gm.; sucrose, 32 gm.; cottonseed oil, 30 gm., and salt mixture,

[4] Method: 25 pounds of commercial casein are treated with 30 liters of distilled water acidified with 0.25% glacial acetic acid (60 cc.). The mixture is hand stirred frequently over a 48-hour period with constant agitation maintained by a stream of air led to the bottom of the 12½-gallon container. The acidified water is then poured off through a multi-layered gauze screen. Further extraction is carried on during the next 48 hours by adding 20 liters of 95% alcohol, with frequent stirring. The alcohol is decanted as above. The casein is then spread thinly over a large surface area to dry. Drying may be accelerated by currents of air generated by electric fans.

12 gm. This supplied a daily total of 2535 calories. The cornmeal, peas and salt mixture were diluted with 3 volumes of water and cooked for 2 hours in an open steam cooker. The other ingredients were then added and thoroughly mixed.

Using this diet, canine blacktongue was produced in forty-five dogs, the initial attack appearing between the fifteenth and eighty-seventh days, with an average of 44 days. A rough estimate of the food consumption was charted each day and the animals were weighed twice weekly. Mouth lesions were preceded by a decline of appetite and loss in weight. The first local signs were reddening of the gums or buccal membranes with increased salivation. Smears from the gums at this stage, stained with gentian violet, showed large numbers of cocci, vibrios, fusiform bacilli and spirochetes (Smith, Persons and Harvey, '37). Soft stools usually appeared early and, as the oral lesions progressed rapidly to necrosis, there was often a bloody diarrhea. The weight usually dropped precipitously, necessitating frequent examinations and daily weighing.

About 5% of the animals slowly starved themselves over a period of 3 to 4 months before developing acute blacktongue and had to be discarded, because extreme emaciation seemed to interfere with responses to specific treatment.

Occasionally, after the appearance of typical early signs there was a spontaneous remission associated with self-starvation and temporary improvement of the mouth lesions, although there was never any gain in weight. The appearance of mouth lesions could not, therefore, be used as the only criterion for the institution of treatment. We have used the following criteria: 1) reddening of the buccal membranes, 2) anorexia, 3) increased salivation, 4) marked weight loss. A later sign is the appearance of a purulent oral discharge, and treatment at this stage is often ineffectual. Frequent examinations of the dogs, however, enabled us to produce 152 definite attacks of blacktongue in the forty-five dogs used in the study.

TREATMENT

In both pellagra and experimental blacktongue acute manifestations progress rapidly but usually respond to adequate treatment within 3 to 5 days. No supplement requiring a long period of administration would be of practical value. For this reason the time for administering the therapeutic supplement was limited to 10 days, and a dosage was judged entirely by its adequacy, under these conditions, when given for 10 days or less. The criteria used for success of the supplemental treatment were: 1) restoration of the appetite, 2) cure of the oral lesions, 3) rapid gain in weight back to the approximate normal for the particular dog, 4) protection against the next attack of blacktongue for 20 days or more.

The evidences of inadequacy of a supplement were: 1) failure to improve the mouth or temporary improvement followed by a relapse as soon as treatment was stopped, 2) persistence of anorexia, 3) a declining weight curve and 4) subsequent restoration of the dog to normal by adequate supplemental treatment. The latter test is necessary because some dogs develop severe infections of the lungs or intestines and succumb regardless of treatment. If a failing dog can be restored to normal by a potent treatment the inadequacy of the test supplement is clear. The results which fall between those we have designated as 'successful' and 'unsuccessful' will be called 'partially successful,' and will be described.

The dogs were kept on the basic deficient diet from the beginning of the experiment until they died or were discarded. Whenever, after proper response to therapy or reconditioning, a dog again developed blacktongue it was used immediately for another therapeutic test. If the previous treatment was unsuccessful or only partially successful, it was necessary to recondition the dog with supplements known to be potent in order to prevent the development of secondary infections. Occasionally in a severely depleted dog a second 10-day course of treatment was necessary before the dog was restored to normal. Any dog failing to respond to test treatment and reconditioning was discarded.

Under these conditions of study usually three to eight tests can be made with each dog. The basic diet seems to be adequate to maintain good health in dogs when the proper amount of curative supplement is added. One dog had eight attacks of blacktongue during a year of testing and was then maintained for 5 months by constant treatment with the liver supplements.

EXPERIMENTS WITH AQUEOUS LIVER EXTRACT GIVEN ORALLY

Ruffin and Smith ('37) found that the daily oral administration of an aqueous preparation, 'Solution liver extract—Valentine' (N.N.R.), derived from 675 gm. of fresh liver was successful in curing twenty out of twenty-two patients with acute pellagra when the patients were being maintained on a basic diet deficient in the P.P. factor of Goldberger. That the basic diet was deficient in the P.P. factor is evidenced by the fact that it regularly produced blacktongue when fed to dogs (Ruffin and Smith, '34).

Three dogs with acute blacktongue were given this aqueous liver extract daily for 10 days, representing a total dose derived from 3560 gm. of fresh liver. A satisfactory result was obtained in all three animals. The chart of the dog which gave the most dramatic response is shown as figure 1. In this, and in subsequent charts, the actual weight of the dog at the time treatment was started is charted at the zero line and the gain or loss in kilograms is recorded during the period of treatment and until blacktongue again appeared.

No attempt was made to determine the minimal curative dose of this crude but effective material.

EXPERIMENTS WITH PARENTERAL LIVER EXTRACTS

Extracts of liver, which were prepared originally for the parenteral treatment of pernicious anemia, have been used successfully in the treatment of clinical pellagra by Boggs and Padget ('32), Ramsdell and Magness ('33), Spies ('33, '35, '37), J. H. Smith ('34), Fouts and Zerfas ('34) and Wylie ('35). These observers gave the parenteral liver extract as a

supplement to a well-rounded diet. Ruffin and Smith ('34, '37) treated twenty-three cases of acute pellagra with parenteral extracts of liver while the patients were being maintained on a basic diet deficient in the P.P. factor. No improvement resulted when the amount of material used was derived

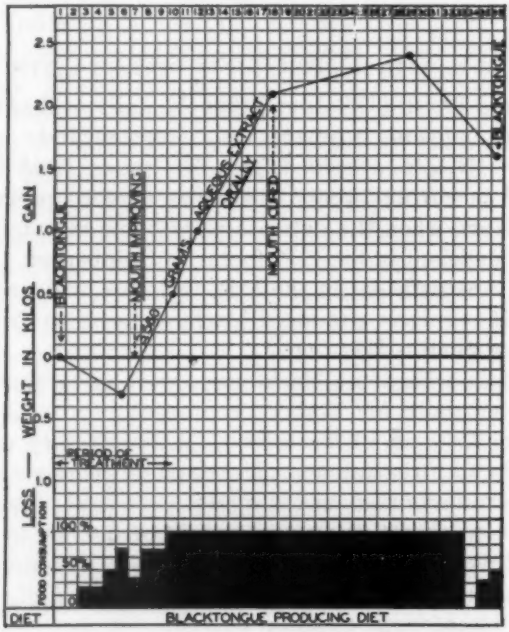

Fig. 1 This figure shows the best one of three tests obtained with Valentine's aqueous extract derived from 3560 gm. of fresh liver. In this and in subsequent figures the animal's weight at the time blacktongue appeared was taken as the zero point and the gain or loss in kilograms charted until the animal again developed blacktongue.

from less than 400 gm. of liver. With doses derived from more than 400 gm. of liver there was some subjective improvement and prompt cure of the acute glossitis, but there was an inconstant effect on the diarrhea and no improvement in the appetite. The most striking observation was the recurrence

of acute symptoms, including an acute glossitis, which followed the exposure of these treated patients to direct sunlight (Smith and Ruffin, '37). No additional improvement resulted from using over ten times the minimal dose. No difference was found in various commercial preparations of parenteral liver extract when they were used in comparable doses. Ruffin and Smith concluded that parenteral liver preparations are only partially effective in the cure of pellagra.

Five dogs with acute blacktongue were given, by mouth, daily for 3 days "Lederle solution liver extract parenteral refined and concentrated" (N.N.R.). The total dose was derived from 300 gm. of liver, or, on the basis of weight, two to three times the partially effective dose for man. The time of treatment was limited to 3 days because dramatic improvement in the acute glossitis always occurred in pellagrins in less than 3 days, when they received this material. There was definite improvement in the oral lesions of the dogs following this treatment but the appetite remained poor, they continued to lose weight and the blacktongue recurred after treatment was discontinued. Four of the five dogs were subsequently cured when given adequate supplemental treatment.

The same experiment was repeated on six dogs with acute blacktongue, except that the dose of extract was given intramuscularly. The temporary improvement in the mouth was more dramatic, and the weight loss less precipitous, but this treatment was also unsuccessful. There was no essential difference between the results of the oral and parenteral methods of administration of this extract. This is in accord with Ruffin and Smith's results in pellagra and in sharp contrast with the effect of the same liver extract in pernicious anemia where the parenteral administration is sixty times as effective as the oral.

Five dogs with acute blacktongue received, intramuscularly, ten times the dose of the Lederle parenteral extract which, in the last experiment, proved ineffective. Of these, three dogs received the entire amount in 3 days and two received the dose over a period of 10 days. There was no difference in the results in the two groups. In this experiment the oral lesions

were promptly cured, the appetite improved somewhat, but the animals gained less than 50% of the weight required for a successful experiment and relapsed after an average interval of 22 days. This treatment was only partially successful.

However, when dogs were treated intramuscularly with the parenteral extract derived from 300 gm. of liver within a 3-day period and orally with the residue [5] derived from 3000 gm. of liver over a 10-day period a dramatic response followed. Early in the experiments it was found that dogs with their first attack of blacktongue, or suffering with a relapse following an unsuccessful test, responded in a dramatic fashion to this treatment. It became evident that this supplement contained a considerable excess of blacktongue curative substances. It was reserved thereafter for reconditioning dogs which were almost moribund as a result of previous unsuccessful tests.

Figure 2 shows the average weight curves of the group of dogs treated with parenteral extracts of liver compared with a standard curve of the average of responses, in eighteen dogs, to this large therapeutic supplement.

EXPERIMENTS WITH LIVER 'RESIDUE'

Ruffin and Smith ('37) gave four patients with acute pellagra the Lederle residue orally for 7 to 10 days. The total dose was derived from an amount of liver which varied from 4376 to 9600 gm. The patients either failed to show any evidence of improvement or improved slightly and relapsed after exposure to sunlight. This was in sharp contrast to the results with Valentine's oral liver extract which produced uniformly satisfactory results in pellagra when given in amounts derived from 4725 to 6750 gm. When the dose of Lederle's liver residue was increased so that the total dose for the treatment was derived from 19,000 to 28,000 gm. of liver it was not only

[5] The addition of 95% ethyl alcohol to an aqueous extract of liver results in a relatively clear supernatant fluid and a dark brown viscid residue. The Lederle Biological Laboratories furnished a supply of this residue concentrated so that 1 cc. was derived from 32 gm. of fresh liver.

effective in pellagra but also in pernicious anemia and sprue. The results with these huge and impractical doses indicate that not all of the hemopoietic material active in pernicious anemia had been removed from this residue.

Ten dogs with acute blacktongue were given by mouth the Lederle liver residue for a 10-day period or a total dose derived from 1000 gm. of liver. The oral lesions progressed,

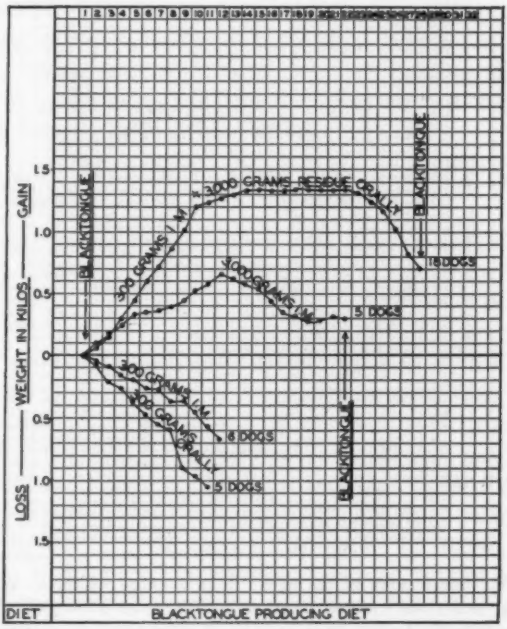

Fig. 2 The lowest curve from the bottom of the figure is the average weight curve of five dogs who were given orally the material derived from 300 gm. of liver in the form of the parenteral fraction which is sold commercially for intramuscular injection in the treatment of pernicious anemia. The second curve from the bottom is an average weight curve of six dogs who received by intramuscular injection the material derived from 300 gm. in the form of the parenteral fraction. The third curve from the bottom gives the average weight curve of five dogs who received by intramuscular injection the same material derived from 3000 gm. of liver. The top curve is the standard satisfactory curve derived by treating eighteen dogs with the parenteral liver fraction derived from 300 gm. (intramuscular injection) and the residue fraction derived from 3000 gm. of liver (orally).

the appetite remained poor and, with but two temporary exceptions, they continued to lose weight. Three of the animals died. The other seven made a satisfactory response when given an adequate supplement. This treatment was, therefore, unsuccessful.

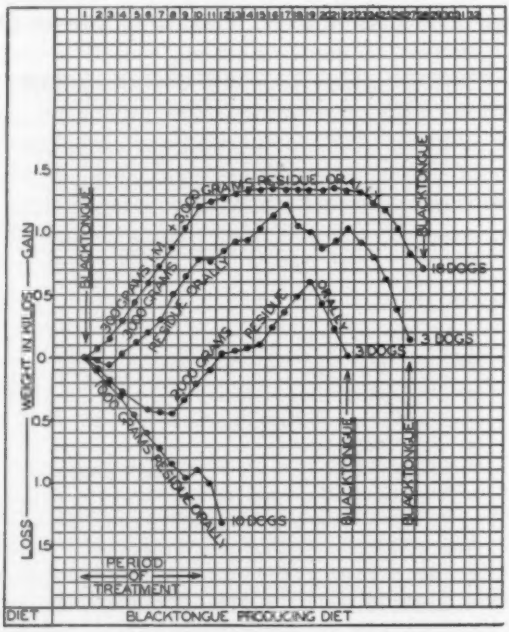

Fig. 3 The lowest curve in the figure is an average weight curve of ten dogs who received orally the residue fraction derived from 1000 gm. of liver. The second curve from the bottom is an average of three dogs who received the residue fraction derived from 2000 gm. of liver. The third curve from the bottom is an average of three dogs who received the residue fraction derived from 3000 gm. of liver. The top curve is the standard curve repeated from figure 2.

In the next experiment the daily dose of the Lederle liver residue was doubled. In three dogs with acute blacktongue the oral lesions remained stationary for 4 to 5 days, the appetite remained unchanged for 6 to 8 days and as seen in figure 3, the animals lost weight steadily for 8 days. The mouth then

began to heal, the appetite improved and the animals gained weight for 10 days but relapsed with blacktongue in an average time of 22 days. This treatment was partially successful.

In a third experiment Lederle liver residue derived from 3000 gm. was used for the total dose in a 10-day period. In three dogs the oral lesions improved slowly, the appetite began to improve after the third or fourth day, and the weight curve rose steadily thereafter. The animals relapsed with blacktongue after an average time of 27 days. This was considered a good response although it differed from the standard response in that there was some delay in healing the mouth and a lag in the weight gain (fig. 3). Drying the residue was found to decrease its curative value.[6]

EXPERIMENTS WITH COMBINATIONS OF PARENTERAL EXTRACTS AND LIVER RESIDUE

Ruffin and Smith ('37) reported four cases of acute pellagra which made dramatic recoveries when the partially effective parenteral liver preparation was supplemented with ineffective amounts of the residue. Our experience with the reconditioning treatment described also pointed to the importance of an investigation of the effects of a combination of fractions derived from liver. It was shown in previous experiments that the dose of parenteral liver derived from 300 gm. was unsatisfactory in eleven dogs (fig. 2). The 1000 gm. dose of the liver residue when given alone was also entirely unsatisfactory in ten dogs (fig. 3).

Five dogs with acute blacktongue received intramuscularly Lederle's parenteral liver extract derived from 300 gm. and orally Lederle's residue derived from 1000 gm. of liver. This combined treatment produced an immediate improvement in the oral lesions, a sharp rise in the weight curve and the animals were protected from blacktongue for an average time of 19 days (fig. 4). This result was striking when compared with

[6] Residue dried in vacuo was furnished by Lederle. One dog with acute blacktongue received a total dose derived from 1500 gm., another that derived from 3000 gm. and a third the dried 'residue' from 6000 gm. of liver. The 6000 gm. dose was found to be partially effective; the smaller doses failed completely.

the effect of the two substances when given separately but must be interpreted as on the border line of adequate supplemental therapy.

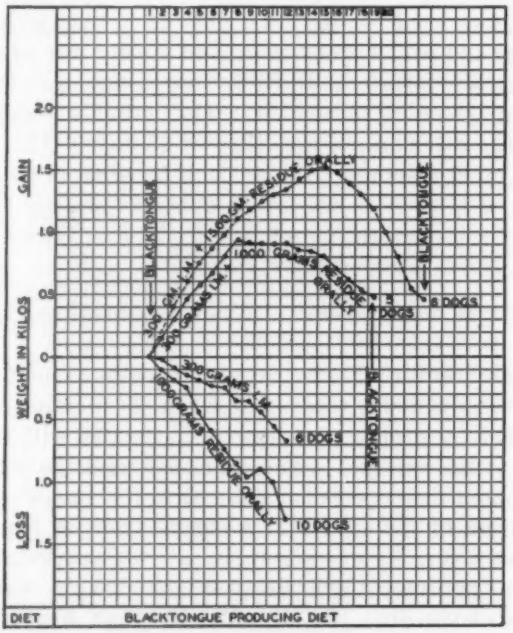

Fig. 4 This figure shows the failure of ten dogs to respond to the liver residue derived from 1000 gm. of liver (curve repeated from fig. 3) and the failure of six dogs to respond to the parenteral fraction derived from 300 gm. of liver (curve repeated from fig. 2) and the dramatic response of five dogs to the combination of these two ineffective doses. The broken curve at the top shows the effect in six dogs of increasing the residue fraction to an amount derived from 1500 gm.

This response to the combined treatment was therefore used as a temporary standard, and an attempt was made to determine which of the two substances was the limiting factor in the experiment. Holding the 1000 gm. dose of liver residue constant, the amount of the parenteral liver was varied. Three dogs with acute blacktongue received intramuscularly the

parenteral liver derived from 30 gm. Four dogs received intramuscularly the parenteral liver derived from 100 gm. Three dogs received intramuscularly the parenteral liver derived from 200 gm. One of the three dogs receiving the 200 gm. dose improved with respect to the oral lesions and gained some weight during the first 10 days of the experiment but then relapsed with blacktongue. None of the other dogs showed any signs of improvement. These results indicated that the dose of the parenteral extract, derived from 300 gm., was the absolute minimum.

With the 1000 gm. dose of the liver residue still held constant, the parenteral liver was doubled. Three dogs with acute blacktongue were given intramuscularly the parenteral liver derived from 600 gm. These animals made no more improvement than the ones which received the parenteral material from only 300 gm. From this we concluded that the parenteral liver extract was not the limiting factor.

Having established the accuracy of the 300 gm. dose of the parenteral liver extract this factor was held constant and the amounts of the oral liver residue varied. One dog received the liver residue derived from 60 gm. A second dog received the liver residue derived from 120 gm. A third dog received the liver residue derived from 175 gm. Four dogs received the liver residue derived from 500 gm. One dog received the liver residue derived from 750 gm. The animal receiving the 750 gm. dose made some improvement, gained weight for 10 days and then relapsed with blacktongue. None of the other dogs showed any improvement. From these results we concluded that the liver residue from 1000 gm. or more was necessary.

It was apparent that the limiting factor must be the liver residue. Five dogs with acute blacktongue were given intramuscularly the Lederle's parenteral liver derived from 300 gm. and the liver residue derived from 1500 gm. These results were very satisfactory. The oral lesions healed promptly, the appetite increased, there was a sharp rise in the weight curve which went above the curve which had been used as a

standard for a successful response (fig. 4). This combination was regarded as the minimum for an entirely satisfactory response.

DISCUSSION

Goldberger and Sebrell ('31) were the first to use liver extracts in the treatment of experimental canine blacktongue. They found that a daily oral dose of 4 gm. of Lilly's 343 dry powdered liver extract (derived from 100 gm. of fresh liver) prevented the development of blacktongue in five dogs and cured four out of five animals with this disease. Rhoads and Miller ('33) were uniformly successful in preventing blacktongue with the same dose of powdered extract used by Goldberger and Sebrell. With this product Miller and Rhoads ('35) succeeded in curing hyperchromic anemia produced by feeding Goldberger's canine-blacktongue-producing diet to pigs. Birch, György and Harris ('35) cured canine blacktongue induced by the Goldberger diet by the daily administration of Lilly's 343 powder derived from 100 gm. of liver but could not cure canine blacktongue produced on a synthetic diet free of B_6 (rat anti-dermatitis factor) until the liver extract was supplemented with cornmeal containing B_6. Fouts, Lepkovsky, Helmer and Jukes ('36) cured two cases of pellagra by administering daily 6 vials (derived from 600 gm.) of Lilly's powdered extract.

Lilly's powdered liver extract 343 is, therefore, effective in curing canine blacktongue and human pellagra. The liver extracts prepared for intramuscular and intravenous use are much more refined and do not produce comparable results either in pellagra (Ruffin and Smith, '37) or in canine blacktongue (Rhoads and Miller, '33). There is some confusion in the literature between Lilly's powdered extract 343 and 'solution of liver extract—Lilly' (N.N.R.) which is sometimes erroneously referred to as Lilly's intramuscular extract 343. This emphasis on the difference in the potency of the two liver extracts should not be construed as a criticism of the excellent clinical results obtained in the treatment of pellagra with intramuscular and intravenous preparations of liver by

Ramsdell and Magness ('33), Spies ('33, '35, '37), Fouts and Zerfas ('34), J. H. Smith ('34), Wylie ('35) and Haden ('36) since their patients were receiving at the same time a well-rounded hospital diet which contains relatively large amounts of additional pellagra curative substances.

SubbaRow, Jacobson and Fiske ('35) prepared a very refined extract of liver designated as '266' which was remarkably effective in pernicious anemia. This product was very effective in curing the mouth lesions of dogs with experimental blacktongue. Koehn and Elvehjem ('36) showed that the chick 'filtrate factor' prepared from liver would cure blacktongue in dogs. Fouts, Lepkovsky, Helmer and Jukes ('36) cured three pellagrins with a similar preparation yet Jukes ('37) suggests that the chick factor is distinct from the P.P. factor because there is some difference between the distribution of the chick factor and the P.P. factor in various food substances. Dann ('37)[7] showed that nicotinic acid (Eastman) was not effective in curing chick dermatitis or rat dermatitis and his results have been confirmed by Fouts, Helmer, Lepkovsky and Jukes ('37) who used synthetic nicotinic acid.

The experiments reported in the present communication indicate that there are two factors in liver necessary for the cure of experimental canine blacktongue. In contrast to this Margolis, Margolis and Smith,[8] working in this laboratory, have made over forty tests with nicotinic acid by the same standard method which we used for the liver fractions and obtained entirely satisfactory results on dogs with their first and second attacks of blacktongue. If the presence of nicotinic acid in either or both of the fractions of liver we have used were the sole factor involved in the production of this standardized 'cure' of blacktongue, there should be a simple summation relationship between the amounts of each fraction necessary to produce a supplement adequate for cure. Our

[7] Presented before the American Dietetic Association, Richmond, Va., October 20, 1937.

[8] Margolis, L. H., G. Margolis and S. G. Smith Unpublished data.

data do not reveal any such simple summation effects. It has been established that aqueous and powdered extracts of whole liver contain curative material for pellagra and blacktongue, but that liver extracts purified for parenteral use are, even in massive dosage, only partially effective when the diet is carefully controlled. The results of Ruffin and Smith ('37) in pellagra, and our present data in experimental canine blacktongue, indicate that relatively small amounts of this ineffectual parenteral fraction of liver may replace considerable amounts of the residue remaining after fractionation of liver extract. In other words, the parenteral extract apparently can replace a part but not all of the residue material in a curative supplement, a finding inconsistent with the possibility that nicotinic acid, as the sole factor involved, is present in therapeutically significant amounts in either or both fractions of liver.

Two further possibilities remain to be considered. The first, that the curative substances in liver are not related to nicotinic acid, seems unlikely because the latter has been found in liver. The second possibility involves the assumption that there may be two substances present in aqueous extracts of liver, that they are partially separable by the methods of fractionation in use, and that both must be administered in order to supply a nicotinic acid complex necessary for the cure of pellagra and experimental blacktongue.

SUMMARY AND CONCLUSIONS

By careful observation, under controlled conditions, it has been possible to produce repeated attacks of experimental canine blacktongue and to compare the therapeutic effects of various dietary supplements derived from liver. Definite criteria for successful therapeutic results from a supplement administered for 10 days or less have been outlined, and the effects of various combinations of supplements tested. The method is suitable for the assay of blacktongue curative potency of any substance which can be administered to dogs.

Varying the supplemental treatment in 152 attacks of experimental blacktongue in forty-five dogs gave results from which we conclude: a) that simple aqueous extracts of liver are potent in 'cure' of the condition; b) that extracts of liver prepared for parenteral use in pernicious anemia are only partially successful, even in huge dosage; c) that the residue from preparation of these parenteral liver extracts is potent in a dosage comparable to the amount of the same material which is potent for pernicious anemia and sprue in man; d) that parenteral extracts, in amounts which are alone ineffectual, may replace considerable amounts of the residue in combinations which produce successful therapeutic results.

These results are not consistent with the possibility that nicotinic acid, which is also effective in experimental canine blacktongue, is present in therapeutically significant amounts in either or both fractions of liver tested. The results suggest that two substances, partially separable by the methods of fractionation in use, are present in liver, and that both are necessary for the elaboration of some compound (probably a nicotinic acid complex) which is potent in the cure of experimental blacktongue in dogs and pellagra in man.

LITERATURE CITED

BIRCH, T. W., P. GYÖRGY AND L. J. HARRIS 1935 The vitamin B_2 complex. Differentiation of the antiblacktongue and the 'P.P.' factors from lactoflavin and vitamin B_6 (so-called 'rat pellagra' factor). Biochem. J., vol. 29, p. 2830.

BOGGS, T. R., AND PAUL PADGET 1932 Pellagra: analysis of 102 cases. Bull. Johns Hopkins Hosp., vol. 50, p. 21.

DANN, W. J. 1936 The vitamin G complex; the non-identity of rat dermatitis due to vitamin B_6 deficiency and the dermatitis of human pellagra. J. Nutrition, vol. 11, p. 451.

———— 1937 Nicotinic acid and vitamin B. Science, vol. 86, p. 616.

———— 1938 Animal deficiency diseases related to pellagra. J. Am. Diet. Assn., vol. 14, p. 157.

DANN, W. J., AND Y. SUBBAROW 1938 Vitamin G complex. Differentiation of the rat dermatitis factor and the chick dermatitis factor from nicotinic acid. J. Nutrition. (In press.)

ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG AND D. W. WOOLLEY 1937 Relation of nicotinic acid and nicotinic acid amide to canine blacktongue. J. Am. Chem. Soc., vol. 59, p. 1767.

ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG AND D. W. WOOLLEY 1938 The isolation and identification of the anti-blacktongue factor. J. Biol. Chem., vol. 123, p. 137.

FOUTS, P. J., AND L. G. ZERFAS 1934 Pellagra in Indiana and its treatment with liver extracts. J. Ind. Med. Assn., vol. 27, p. 196.

FOUTS, P. J., S. LEPKOVSKY, O. M. HELMER AND T. H. JUKES 1936 Successful treatment of human pellagra with the 'filtrate factor.' Proc. Soc. Exp. Biol. and Med., vol. 35, p. 245.

FOUTS, P. J., O. M. HELMER, S. LEPKOVSKY AND T. H. JUKES 1937 Treatment of human pellagra with nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 405.

GOLDBERGER, J., AND W. H. SEBRELL 1930 The blacktongue preventive value of Minot's liver extract. Pub. Health Reports, vol. 45, p. 3064.

HADEN, R. L. 1936 Multiple specific nutritional deficiency disease in the adult. J. Am. Med. Assoc., vol. 106, p. 261.

HARRIS, L., AND A. HASSAN 1937 Nicotinic acid and the pellagra preventing ('P.P.') vitamin. Lancet, vol. 233, p. 1467.

JUKES, T. H. 1937 Further observations on the assay, distribution, and properties of the filtrate factor. J. Biol. Chem., vol. 117, p. 11.

KOEHN, C. J., JR., AND C. A. ELVEHJEM 1936 Studies on vitamin G (B_2) and its relation to canine blacktongue. J. Nutrition, vol. 11, p. 67.

RAMSDELL, R. L., AND W. H. MAGNESS 1933 Parenteral liver extract therapy in treatment of pellagra. Am. J. Med. Sci., vol. 185, p. 568.

MILLER, D. K., AND C. P. RHOADS 1935 The experimental production of loss of hematopoietic elements of the gastric secretion and of the liver in swine with achlorhydria and anemia. J. Clin. Invest., vol. 14, p. 153.

RHOADS, C. P., AND D. K. MILLER 1933 The production in dogs of chronic blacktongue with anemia. J. Exp. Med., vol. 58, p. 585.

———— 1935 Vitamin B_2 (G) and canine blacktongue. Science, vol. 81, p. 159.

RUFFIN, J. M., AND D. T. SMITH 1934 The treatment of pellagra with certain preparations of liver. Am. J. Med. Sci., vol. 187, p. 512.

———— 1937 A clinical evalution of the potency of various extracts of liver in the treatment of pellagra. So. Med. J., vol. 30, p. 4.

SMITH, S. G. 1932 A new symptom complex in vitamin G deficiency in rats. Proc. Soc. Exp. Biol. and Med., vol. 30, p. 198.

———— 1938 Etiology of sebaceous gland atrophy in the rat in avitaminosis. J. Nutrition, vol. 15, p. 45.

SMITH, S. G., G. MARGOLIS AND L. H. MARGOLIS 1938 Effect of autoclaving on the vitamin potency of nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 38, p. 251.

SMITH, S. G., AND D. H. SPRUNT 1935 Pathological skin changes in the tail of the albino rat on a diet deficient in vitamin G. J. Nutrition, vol. 10, p. 481.

SMITH, D. T. 1938 Present knowledge of pellagra: clinical studies. J. Am. Diet. Assn., vol. 14, p. 168.

SMITH, D. T., AND J. M. RUFFIN 1937 Effect of sunlight on the clinical manifestations of pellagra. Arch. Int. Med., vol. 59, p. 631.

SMITH, D. T., E. L. PERSONS AND H. I. HARVEY 1937 On the identity of the Goldberger and Underhill types of canine blacktongue; secondary fuso-spirochetal infection of each. J. Nutrition, vol. 14, p. 373.

SMITH, D. T., J. M. RUFFIN AND S. G. SMITH 1937 Pellagra successfully treated with nicotinic acid; a case report. J. Am. Med. Assoc., vol. 109, p. 2054.

SMITH, J. H. 1934 The treatment of pellagra. So. Med. J., vol. 27, p. 163.

SPIES, T. D. 1933 The treatment of pellagra by means of parenteral liver extract. Proc. Soc. Exp. Biol. and Med., vol. 31, p. 363.

———— 1935 The treatment of pellagra. J. Am. Med. Assoc., vol. 104, p. 1377.

SPIES, T. D., A. CHINN AND J. B. MCLESTER 1937 Treatment of endemic pellagra. So. Med. J., vol. 30, p. 18.

SPIES, T. D., C. COOPER AND M. A. BLANKENHORN 1937 Therapeutic administration of nicotinic acid in human beings during health and disease. Presented before the Central Society for Clinical Research, Nov. 5.

———— 1938 Nicotinic acid in the treatment of pellagra. J. Am. Med. Assoc., vol. 110, p. 622.

STREET, H. R., AND G. R. COWGILL 1937 The cure of canine blacktongue with nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 547.

SUBBAROW, Y., B. M. JACOBSON AND C. H. FISKE 1935 The separation of the substances in liver which are reticulocytogenic in the guinea pig and which are therapeutically effective in experimental canine blacktongue. New England J. Med., vol. 212, p. 663.

UNDERHILL, F. P., AND L. B. MENDEL 1928 A dietary deficiency canine disease. Further experiments on the diseased condition in dogs described as pellagra-like by Chittenden and Underhill and possibly related to so-called blacktongue. Am. J. Physiol., vol. 83, p. 589.

WYLIE, W. D. 1935 The treatment of pellagra. So. Med. and Surg., vol. 97, p. 14.

A COMPARISON OF SODIUM FLUORIDE IN THE DRINKING WATER WITH SIMILAR LEVELS OF CRYOLITE IN THE DIET ON THE FLUORINE CONTENT OF THE BODY

S. MARCOVITCH AND W. W. STANLEY[1]

Agricultural Experiment Station, University of Tennessee, Knoxville

ONE FIGURE

(Received for publication January 31, 1938)

In a previous publication we showed that the teeth of rats gave higher fluorine values on fluorides in the drinking water than on smilar levels of fluorine as cryolite in the food (Marcovitch, Shuey and Stanley, '37). Fluorine now finds its way into the body with the drinking and cooking water, as spray residues, with the use of bone meal in baby foods, and as natural fluorine in foods. The retention of the fluorine from these different sources undoubtedly varies, and it becomes of importance to evaluate them. In this study we have attempted to follow the rate of fluorine retention in the body from cryolite additions to the diet as compared with similar levels in the drinking water. The results are also compared with the rate of calcium retention in the body from birth to maturity, as studied by Sherman and MacLeod ('25).

METHODS

For the study of fluorine retention, twenty-nine young rats were used. These animals were 28 days old, of uniform size and in good physical condition. A synthetic cryolite, a commercial product composed of finely divided particles suitable

[1] Valuable assistance in feeding the animals was rendered by F. L. MacLeod and Evelyn Utley. We wish also to acknowledge the analytical assistance on the determination of fluorine by R. J. Rowley, of the Aluminum Research Laboratories.

for insecticide purposes, was added to a standard diet (diet B of Sherman and McCampbell) of ten animals at the rate of 4 p.p.m. of fluorine. Another group of nine animals was given the same diet and the fluorine was supplied through the drinking water by the addition of sodium fluoride (Baker's C.P.) at the rate of 4 p.p.m. of fluorine in solution. A third group, consisting of ten animals, was raised on a similar diet but received no added fluorine.

The animals were chloroformed and fluorine determinations were made at birth, 29 days and at 90 days. It was hoped to get the fluorine contents at intervals of 30 days up to 360 days, but this was not possible. The fluorine was determined by the method of Willard and Winter ('33) and double distilled according to Churchill and co-workers ('37) to prevent phosphate interference. The entire body of the animals except the stomach and intestines was analyzed for fluorine. The stomach and intestines were not removed in the newborn young and the 29-day-old rats.

FLUORINE CONTENT OF THE BODY

As shown in table 1, the fluorine content at birth was found to average 4.36 p.p.m. At the end of 29 days the fluorine content was approximately the same, that is, 4.03 p.p.m. For the control animals only 2.09 p.p.m. was present at the end of 90 days. This is shown graphically in figure 1. The course of calcium retention during this same period is also shown in figure 1 from data obtained from Sherman and MacLeod. An inspection of this curve shows that the calcium of the newborn young is low, at which time there is present 0.25% or 2500 p.p.m. At the end of 30 days the calcium increases to 0.71%, while at 90 days it reaches 1.01%.

The ratio of fluorine to calcium at birth equals 1:573. At 30 days the ratio drops to 1:1598, indicating a relatively larger retention of calcium. At 90 days, this divergence becomes still more marked for the ratio becomes 1:4832. There is thus a greater percentage of fluorine present at birth than at any subsequent period.

TABLE 1

Fluorine content of rats fed 4 p.p.m. of fluorine as cryolite in diet as compared with 4 p.p.m. of flourine as sodium fluoride in the drink

NO.	WEIGHT OF RAT	FLUORINE CONTENT	
		mg.	p.p.m.
Newborn			
5173, 5163 } 10 y	20.3	0.08	2.7
4312, 4309 } —9 y	22.6	0.05	2.2
4303, 4309 } —6 y	20	0.18	9.0
Average	23.60	0.10	4.36
Total average	2.84	0.012	4.36
29 days old (No added fluorine)			
Males			
5477	70.3	0.23	3.2
5446	70.4	0.28	4.0
5453	64.0	0.48	7.4
5434	63.6	0.19	2.9
Average	67.07	0.29	4.38
Females			
5473	59.1	0.14	2.3
5444	80.6	0.21	2.5
5437	57.7	0.18	3.1
5509	36.1	0.19	5.1
5480	61.3	0.36	5.8
Average	58.9	0.22	3.76
Total average	62.56	0.25	4.03
90 days old 4 p.p.m. of fluorine as cryolite in diet			
Males			
5451	274.6	1.61	5.9
5441	313.8	2.04	6.3
5431	262.0	1.94	7.4
5447	276.1	1.88	6.8
5454	250.5	1.88	7.4
5459	170.2	1.09	6.4
Average	257.8	1.73	6.68

TABLE 1—*Continued*

NO.	WEIGHT OF RAT	FLUORINE CONTENT	
		mg.	p.p.m.
Females			
5467	167.8	1.44	8.6
5464	198.3	1.47	7.4
5472	196.3	1.35	6.9
5435	190.8	1.46	7.6
Average	188.3	1.43	7.46
Total average	230.4	1.61	7.07
90 days old 4 p.p.m. of fluorine as sodium fluoride in drink			
Males			
5447	230.3	3.00	13.0
5455	271.2	2.96	10.9
5442	346.2	4.02	11.6
5432	271.2	3.06	11.3
Average	279.7	3.25	11.7
Females			
5462	172.7	2.36	13.7
5430	187.9	2.64	14.1
5466	177.3	2.46	13.9
5474	175.4	2.58	14.7
5450	173.2	2.40	13.9
Average	177.3	2.48	14.06
Total average	222.8	2.83	13.02
90 days old Control—no fluorine added to diet 'B'			
Males			
5430	287.0	0.48	1.7
5443	321.7	0.33	1.0
5448	313.8	0.82	2.6
5456	260.2	0.26	1.0
Average	295.6	0.47	1.57
Females			
5452	175.0	0.20	1.1
5465	184.3	0.56	3.0
5475	168.3	0.38	2.3
5463	156.8	0.65	4.1
5469	270.2	0.35	1.3
5440	188.3	0.52	2.8
Average	190.4	0.44	2.43
Total average	232.5	0.45	2.09

In a series of rats fed 4 p.p.m. of cryolite in the diet, beginning on the twenty-ninth day and continued to 90 days of age, the average fluorine content of the body showed 7.07 p.p.m. When sodium fluoride was given in the drinking water containing 4 p.p.m. of fluorine, a figure of 13.02 p.p.m. of body fluorine was found. According to these figures and those obtained for teeth (Marcovitch, Shuey and Stanley, '37), the body stores fluorine nearly twice as fast when the animal is

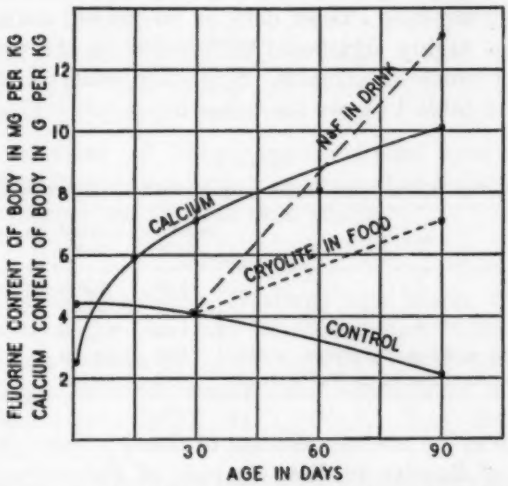

Fig. 1 Comparison of 4 p.p.m. of fluorine in drink and a similar level of fluorine as cryolite on the fluorine content of body. The curve for fluorine and calcium retention in the control animals is also shown.

fed dissolved fluorine in the drinking water as on cryolite at the same level in the food. When the above figures are plotted as shown in figure 1, the rate of fluorine retention with the cryolite additions parallels the rate of calcium retention. The rate of fluorine retention caused by the fluorine in the drinking water is much greater than the rate of calcium retention. In this connection, it is interesting to note that cryolite at 4 p.p.m. of fluorine will not produce striations in the teeth, while sodium fluorine at 4 p.p.m. of fluorine in the

drinking water will produce striations. It appears, therefore, if the fluorine-calcium ratio can be kept below 1:1500, no mottling of the teeth or striations should take place on an adequate diet.

Sherman and MacLeod ('25) contend that females have a higher percentage of calcium in the body than males of the same age which had received the same food. Statistical analysis of their data show no significant differences for sexes of 15-day-old rats and only slight differences for 30- and 60-day-old rats. Their data on 90-day-old animals, however, show highly significant differences in the per cent of calcium in females and males. Statistical analysis of our data as given in table 1 shows the following points: 1) significant

TABLE 2

Fluorine-calcium ratios in the body of the rat at different ages

AGE	FLUORINE P.P.M.	CALCIUM P.P.M.	FLUORINE-CALCIUM RATIO
At birth	4.36	2,500	1: 573
29 days	4.03	6,900	1: 1598
90 days	2.09	10,100	1: 4832
90 days (4 p.p.m. fluorine as cryolite in diet)	7.07	10,100	1: 1428
90 days (4 p.p.m. of fluorine as NaF in drink)	13.02	10,100	1: 749

difference in the average amount of fluorine found in rats fed 4 p.p.m. of fluorine in diet, 4 p.p.m. of fluorine in drinking water and control rats; 2) significant difference in the amount of fluorine found in males and females upon fluorine additions in diet or in drinking water. No significant difference was found in the average amount of fluorine in the sexes in either of the control groups (29- and 90-day-old rats). 3) The fluorine content of the two control groups (29- and 90-day-old rats) showed only slightly significant differences (odds of 20 to 1).

EXCRETION

Exactly what proportion of the ingested fluorine is retained in the body of the rat and how much is excreted is still unknown. Since the control animals showed a fluorine content

of 2.09 p.p.m., there must have been present in the diet about 0.7 p.p.m. of fluorine, which appears to have been all retained. Only about half of the cryolite additions were retained, whereas practically all of the fluorine in the drink as sodium fluoride was retained.

It appears that during active bone formation the ingested fluorine is largely retained. In this connection the fluorine determination of a few samples of human urine is of interest, which we obtained as follows:

	p.p.m.
Adult individual (1)	1.8
Adult individual (2)	1.9
Boy (6 years old)	0.37
Boy (7 years old)	0.48

A large number of determinations would have been desirable. But from these few, it appears that children retain more and excrete less fluorine than adults.

DISCUSSION

According to Roseberry, Hastings and Morse ('31), the calcium salts of the bone may be represented by the general formula of an apatite $CaX_2 \cdot N(Ca_3(PO_4)_2)$ where X may be F or $\frac{1}{2}$ CO_3. In other words, bone consists of a mixture of carbanoapatite $CaCO_3 \cdot N(Ca_3(PO_4)_2)$ and fluorapatite $CaF_2 \cdot N(Ca_3(PO_4)_2)$.

At birth the newborn rat possesses a fluorine-calcium ratio near to 1:573. As in the case of iron, the percentage of fluorine in the body of the rat is higher at birth than at maturity. At 29 days old, the fluorine ratio is still comparatively high. It appears that the fluorine in rat's milk is very efficiently utilized since milk in general is low in fluorine. In order to maintain this same utilization or rate of storage after weaning, additional fluorine would be necessary such as that supplied by 4 p.p.m. of fluorine as cryolite.

The human infant at birth possesses about 1 ounce of calcium or 10 mg. per gram of body weight. The newborn rat has 2.5 mg. of calcium per gram of body weight. Figures on the fluorine content of the human infant are not available,

but since the human infant has four times as much calcium per unit of weight as the rat, there exists the possibility that fluorine content per unit of weight may be four times as great in the human infant.

In evaluating the true effect of dissolved fluorine as it occurs in the well waters of localities of chronic endemic dental fluorosis, it should be noted that more fluorine is ingested in drinking than in similar levels of fluorine in the diet. The relation of foods cooked in water containing toxic amounts of fluorides must also be given consideration. Had we employed a cooked diet instead of the customary raw diet for the rats, the fluorine retention of dissolved fluorine would be several times greater than on fluorine as cryolite in the diet.

In our work we employed cryolite in all of the diet at the rate of 4 p.p.m. of fluorine. Although this amount is lower than any used previously, it is still far greater than any possible consumption of cryolite on sprayed food in practice. Assuming that 10% of the diet is sprayed with cryolite, a concentration of 4 p.p.m. in the total diet would be equivalent to 40 p.p.m. as spray residues. Such high figures would not be possible on sprayed food. A study of the effect of 1 p.p.m. of fluorine as cryolite in the total diet would furnish information showing the relation of fluorine retention of spray residues. Even 1 p.p.m. of fluorine would be equivalent to 10 p.p.m. as spray residues which represents the upper limits that may be found.

SUMMARY

1. Rats on 4 p.p.m. of fluorine in drinking water as sodium fluoride retain nearly twice as much fluorine in the body as on 4 p.p.m. of fluorine as cryolite in the diet. This is due in part to the greater consumption of fluorine in the drinking water, but no doubt solubility is also a factor. In practice fluorine-bearing waters are also employed in cooking, and if we had employed a diet cooked in fluorine-containing water, the retention would have been still greater.

2. The females showed a significantly greater percentage of fluorine in their bodies than the males when given fluorine additions to the diet or drink.

3. No significant differences were noted in the fluorine of the body in the sexes of the control groups (29 days to 90 days).

LITERATURE CITED

CHURCHILL, H. V., R. W. BRIDGES AND R. J. ROWLEY 1937 Interference of phosphorus in the determination of fluorine. Ind. and Eng. Chem., Anal. Ed., vol. 9, p. 222.

MARCOVITCH, S., G. A. SHUEY AND W. W. STANLEY 1937 Cryolite spray residues and human health. Tenn. Agr. Exp. Sta. Bul. 162.

SHERMAN, H. C., AND F. L. MACLEOD 1925 The calcium content of the body in relation to age, growth and food. J. Biol. Chem., vol. 64, p. 429.

ROSEBERRY, H. H., A. B. HASTINGS AND J. K. MORSE 1931 X-ray analysis of bone and teeth. J. Biol. Chem., vol. 90, p. 395.

WILLARD, H. H., AND O. B. WINTER 1933 Volumetric method for determination of fluorine. Ind. and Eng. Chem. Anal. Ed., vol. 5, p. 7.

DIFFERENTIATION OF THE RAT DERMATITIS FACTOR AND THE CHICK DERMATITIS FACTOR FROM NICOTINIC ACID [1,2]

W. J. DANN

Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina

AND

Y. SUBBAROW

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

TWO FIGURES

(Received for publication February 21, 1938)

During the course of an attempt to isolate the various active components of the vitamin B_2 complex from crude aqueous extract of liver, nicotinic acid was separated from fractions having high vitamin B_6 activity early last year. This substance was tested for rat dermatitis curative activity and for chick dermatitis preventive activity and found to possess neither. Following the publication of Elvehjem et al. ('37), who found that it would cure canine blacktongue, nicotinic acid was also tested in blacktongue and in confirmation of the results of Elvehjem et al. was found to be curative.

[1] This paper is no. 12 in a series of coordinated studies on pellagra in man and associated deficiency diseases of animals from the Duke University Medical School, with the cooperation of Dr. Y. SubbaRow of the Department of Biological Chemistry, Harvard Medical School.

[2] We wish to express our thanks to the Rockefeller Foundation, to the Milton Fund of Harvard University and to the Proctor Fund of Harvard Medical School for grants toward the expenses of this work; to the Lederle Laboratories, Pearl River, New York, for assistance in the preparation of liver extracts; to Merck and Company for the gift of pure vitamin B_1; to the Winthrop Chemical Company for the gift of pure riboflavin; and to Miss M. Hess for her skilled assistance in the care of the animals.

A preliminary account of these findings has been published (Dann, '37) and a detailed description of the observations is given below.

EXPERIMENTAL

Isolation of nicotinic acid amide from liver extract. The starting point was the partially purified liver extract described by SubbaRow, Jacobson and Fiske ('36). This extract was treated with fuller's earth as described by SubbaRow, Jacobson and Prochownick ('36), and the fuller's earth holding the adsorbate from 5 kg. of liver was eluted with 700 cc. of 0.1 N sodium hydroxide. The eluate was immediately neutralized with hydrochloric acid and treated with 20 gm. of charcoal. After filtration the charcoal was eluted with hot 60% ethyl alcohol. The alcoholic eluate was concentrated to 50 cc. and treated with 500 cc. of 95% ethyl alcohol and 500 cc. of ether; the mixture was left in the cold room for 24 hours and then filtered. The filtrate was concentrated to 20 cc. and diluted to 100 cc. with water, made alkaline with sodium carbonate and extracted three times with 100 cc. of iso-amyl alcohol. The amyl alcohol extract was concentrated under vacuum with repeated additions of water to remove the amyl alcohol. To the final aqueous concentrate was added saturated flavianic acid, which brought down a crystalline precipitate. The precipitate recrystallized readily from 60% ethyl alcohol; the yield was 280 mg. of flavianate from 5 kg. of liver. Melting point was 254° to 255°C. (uncorr.); M.P. of flavianate of synthetic nicotinic acid amide, 255° to 256°C. (uncorr.). There was no depression of melting point on mixing the two specimens. The flavianic acid was removed and the hydrochloride was crystallized. Combustion gave C, 45.2%; H, 4.7%; and N, 17.5%. Calculated values for $C_6H_7ON_2Cl$ are C, 45.41%; H, 4.45%; and N, 17.68%.

Preparation of liver extract fraction no. 122. The 95% ethyl alcoholic filtrate from Cohn's fraction G (Cohn et al., '28) was diluted with 80% ethyl alcohol until the volume reached 20 cc. for each 100 gm. of liver. Five-tenths gram of norit

charcoal per 100 gram of liver was added and the mixture stirred for ½ hour. The charcoal was filtered off and washed, then eluted twice with hot 60% ethyl alcohol. The eluate was concentrated until 1 cc. represented 100 gm. of liver.

Effect of nicotinic acid upon rat dermatitis. Albino rats from four separate litters aged from 21 to 28 days were housed in screened floor cages and given a diet composed of sucrose 67%, Crisco 3%, crude casein 20%, powdered agar 2%, B D H salt mixture 8%. Each rat was also given from a medicine dropper 20 γ of vitamin B_1 daily, 40 γ riboflavin twice weekly and 40 mg. cod liver oil twice weekly. After development of the characteristic dermatitis of vitamin B_6 deficiency five of these rats were treated with nicotinic acid, receiving either 1 mg. or 2 mg. daily, and littermates were used as negative controls or as positive controls. The positive control rats were given daily doses of a partially purified fraction (no. 122) from aqueous liver extract, known from previous tests to be active in curing rat dermatitis. The results of the experiment are set out in figure 1 which shows that in the doses given nicotinic acid neither cured the dermatitis nor induced growth. The possibility of the given doses of nicotinic acid exerting a toxic effect which might obscure its activity in curing the dermatitis has been excluded by observations on the toxicity of nicotinic acid (McCrea and Dann, '37). Normal rats were shown to tolerate far larger doses of nicotinic acid than those used in the experiment described above without any outward symptoms except a transient indication of physical discomfort immediately after the large doses, which were injected intra-peritoneally.

In a second test of nicotinic acid in treatment of rat dermatitis a number of the rats received a daily dose of only 0.1 mg. of nicotinic acid and others received 1.0 mg. daily. The results are collected in table 1. Again it was found that nicotinic acid did not cure the rat dermatitis. From these results it appears that nicotinic acid is not the rat anti-dermatitis factor (vitamin B_6).

Effect of nicotinic acid upon chick dermatitis. In a preliminary experiment to determine the activity of nicotinic acid in the prevention of chick dermatitis a batch of White Leghorn chicks was placed at hatching on an adequate diet of natural foodstuffs. On the sixth day the regimen was changed by substituting the basal diet described by Lepkovsky and Jukes

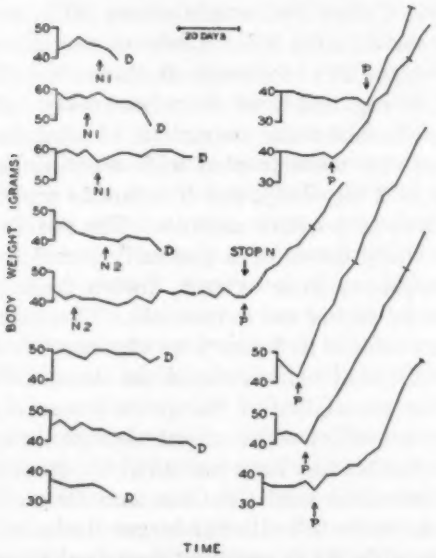

Fig. 1 Weight curves of rats in which severe dermatitis had been induced by feeding a diet devoid of vitamin B_6. Each curve begins with the day on which the characteristic dermatitis first appeared in severe form. N indicates the point at which administration of nicotinic acid was begun (N 1 signifies 1 mg. daily; N 2 signifies 2 mg. daily); P indicates the point at which administration of a known curative fraction was begun; D indicates death of the animal. A crossbar on the curve indicates the point at which the dermatitis had healed.

('35) for the adequate diet and on the nineteenth day the chicks were sufficiently depleted to commence the experiment although none had developed dermatitis at that time. The chicks were divided into groups of nine and additions made to the basal diet as follows: group 1, crude aqueous liver extract derived from 300 gm. liver to each kilo of diet; group 2,

0.5 gm. of nicotinic acid per kilo of diet; group 3, no addition. This regimen was maintained for 2 weeks at the end of which all chicks in group 1 had survived and seven remained normal. Six members of group 2 had survived and all had moderately severe dermatitis; and seven of group 3 survived, all with moderately severe dermatitis. The data for each group are given in table 2. Weighings were made twice weekly and each figure recorded is the mean of the weight of all chicks surviving on the given weighing day.

TABLE 1

The treatment of rat dermatitis with nicotinic acid. Note that the last three rats used as positive controls were dosed with fraction no. 122 after they had been treated ineffectually with nicotinic acid

Rat	*Daily dose*	*Result of treatment*
22.13	0.1 mg. nicotinic acid	Dermatitis not cured in 38 days
22.15	0.1 mg. nicotinic acid	Died after 5 days
22.16	0.1 mg. nicotinic acid	Dermatitis not cured after 38 days
22.25	0.1 mg. nicotinic acid	Dermatitis not cured after 38 days
22.27	0.1 mg. nicotinic acid	Dermatitis not cured after 38 days
22.28	0.1 mg. nicotinic acid	Dermatitis not cured after 38 days
22.28	0.1 mg. nicotinic acid	Died after 9 days
22.33	0.1 mg. nicotinic acid	Died after 39 days, dermatitis not cured
22.35	0.1 mg. nicotinic acid	Died after 40 days, dermatitis not cured
22.43	0.1 mg. nicotinic acid	Dermatitis not cured after 38 days
22.31	1 mg. nicotinic acid	Died after 18 days, dermatitis not cured
22.37	1 mg. nicotinic acid	Died after 12 days, dermatitis not cured
22.39	1 mg. nicotinic acid	Died after 36 days, dermatitis not cured
22.41	1 mg. nicotinic acid	Died after 9 days, dermatitis not cured
22.27	2 drops fraction 122	Dermatitis completely cured in 17 days
22.28	2 drops fraction 122	Dermatitis completely cured in 24 days
22.43	2 drops fraction 122	Dermatitis completely cured in 17 days

This observation was repeated with different amounts of nicotinic acid. Barred Plymouth Rock chicks were used after receiving an adequate diet for 7 days followed by the basal diet for 8 days. Nicotinic acid was incorporated in the diet at two levels of 100 mg. and 1 gm. respectively per kilo of diet for 2 different groups. The results are given in table 2, and it is concluded that nicotinic acid has no activity in the prevention of chick dermatitis and is therefore distinct from the chick dermatitis preventive factor.

TABLE 2

Test of nicotinic acid for preventive activity in chick dermatitis

A. First test, with White Leghorn chicks

Group	1	2	3
Number in group	9	9	9
Addition to basal diet	Aqueous liver extract = 300 gm. of liver per kg. of diet	500 mg. nicotinic acid per kg. of diet	None
Mean body wt. for group	gm.	gm.	gm.
after 0 days	62	62	62
after 4 days	79	64	70
after 7 days	96	67	72
after 10 days	109	67	73
after 15 days	143	73	79
Number of survivors	9	7	6
Condition	Two had slight dermatitis; all others normal	All had moderately severe dermatitis	All had moderately severe dermatitis
Mean wt. gain in 15 days	81	11	17

B. Second test, with Barred Plymouth Rock chicks

Group	1	2	3	4
Number in group	10	11	11	10
Addition to basal diet	Aqueous liver extract = 300 gm. of liver per kg. of diet	100 mg. nicotinic acid per kg. of diet	1 gm. nicotinic acid per kg. of diet	None
Mean body wt. for group	gm.	gm.	gm.	gm.
after 0 days	59	60	60	59
after 4 days	76	66	63	62
after 7 days	90	68	69	63
after 11 days	111	72	76	66
after 14 days	126	76	80	70
Number of survivors	10	10	8	6
Condition	One had slight dermatitis; all others normal	All had severe dermatitis	All had severe dermatitis	All had severe dermatitis
Mean wt. gain in 14 days	67	16	20	11

Effect of nicotinic acid upon canine blacktongue. For a test of the activity of nicotinic acid in blacktongue the curative method of Harvey et al. ('38) was used. The only departures from the procedure described by them were: 1) the use in the

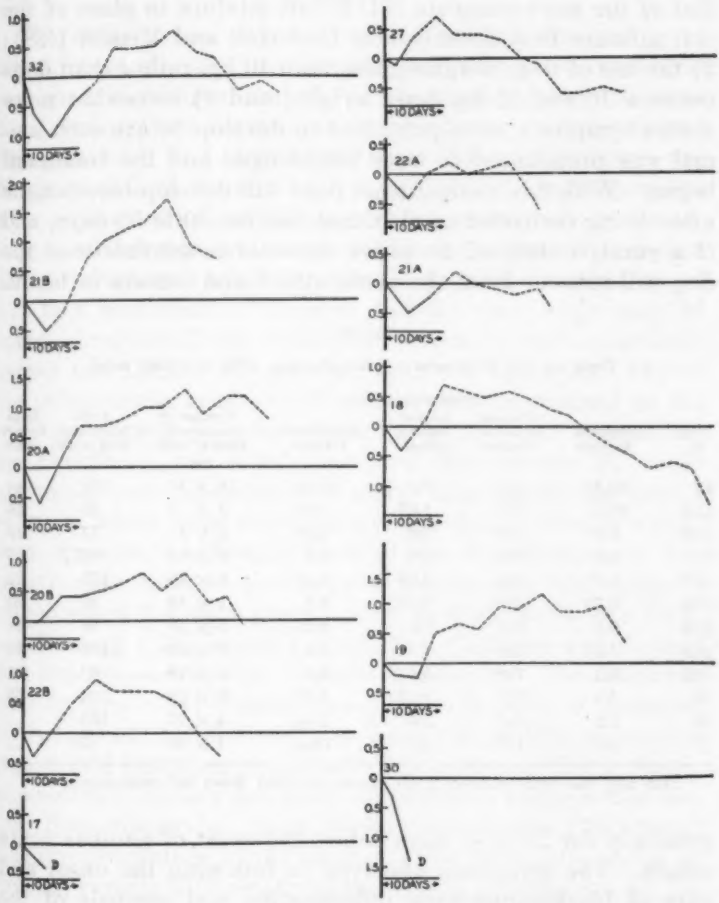

Fig. 2 Weight curves of dogs with blacktongue. Each curve begins on the day when the acute attack of blacktongue developed, and the body weight at that time is made the base line for the curve and marked zero. Each curve continues until the development of the next acute attack; a solid line indicates the persistence of the symptoms and a broken line indicates freedom from the symptoms of blacktongue. Each dog was dosed for a period beginning with the first day charted; for details of dosing consult table 3. Ordinates graduated in kilograms.

diet of the more complete B D H salt mixture in place of the salt mixture first described by Underhill and Mendel ('28); 2) the use of dogs weighing less than 10 kg. rather than dogs between 10 and 15 kg. body weight; and 3) somewhat more severe symptoms were permitted to develop before each animal was pronounced to have blacktongue and the treatment begun. With this method most dogs will develop blacktongue after being restricted to the basal diet for 30 to 50 days, and if a curative dose of an active material is administered the dog will recover from the acute attack and remain in health

TABLE 3

Data on the treatment of blacktongue with nicotinic acid

	Body weight (kg.)						
Dog no.	Previous highest	At onset of blacktongue	Lowest during attack	Subsequent highest	*Number of doses and sizes of each*	*Total nicotinic acid given*	*Days to next onset*
					mg.		
32	10.45	9.5	8.5	10.25	10 × 10	100	44
21A	10.1	9.0	8.65	9.35	7 × 5	35	28
21B	8.9	8.15	7.6	9.85	5 × 15	75	33
27	8.0	6.95	6.8	7.6	5 × 10	50	40
19[1]	6.7	5.6	5.35	6.75	7 × 25	175	41
22A	9.55	8.95	8.55	9.1	1 × 30	30	26
22B	9.5	8.3	7.9	9.15	5 × 10	50	34
20A	7.75	6.8	6.15	8.1	7 × 25	175	42
20B	8.1	7.65	7.65	8.4	5 × 10	50	38
18	5.9	5.1	4.75	5.85	5 × 25	125	55
30	7.2	6.4	5.0	Died	4 × 25	100	..
17	5.5	4.75	4.3	Died	1 × 25	25	..

[1] This dog was treated orally; all others received doses subcutaneously.

generally for 25 to 45 days before the onset of another acute attack. The symptoms observed in following the onset and cure of blacktongue were inflammation and necrosis of the mucous membranes of the mouth and throat, excessive salivation, loss of appetite, diarrhea and weight changes.

In figure 2 and table 3 are shown the data obtained in treatment of twelve attacks of blacktongue in nine dogs with varying doses of nicotinic acid. There can be no doubt that in suitable doses nicotinic acid is effective in the cure of blacktongue.

DISCUSSION

The observations described above confirm the finding of Elvehjem et al. ('37) that nicotinic acid will cure blacktongue and show in addition that the rat dermatitis factor (vitamin B_6 of György) and the chick dermatitis factor ('filtrate factor' of Lepkovsky and Jukes; 'vitamin B_2' of Elvehjem and Koehn) are both distinct from nicotinic acid. In conjunction with earlier work this finding establishes conclusively the existence of four separate factors within the vitamin B_2 complex. The developments which together lead to this conclusion are due to a multitude of workers, but the main steps may be summarized as follows. Following the demonstration by Goldberger and Lillie ('26) and by Smith and Hendrick ('26) of the independent existence of what is now recognized as the complex vitamin B_2, apart from the antineuritic vitamin B_1, the hypothesis was widely accepted that vitamin B_2 would prevent or cure rat dermatitis, canine blacktongue and human pellagra, which were consequently regarded as strictly analogous diseases. Although doubt of this hypothesis was frequently expressed, no evidence was forthcoming to disprove it categorically for some years. Then György, Kuhn and Wagner-Jauregg ('33) isolated crystalline riboflavin and showed that it possessed 'intense vitamin B_2 activity.' The following year György ('34) demonstrated that riboflavin is essential for rat growth but distinct from the rat dermatitis preventive factor, and Elvehjem and Koehn ('34) showed that a factor preventive of dietary deficiency dermatitis of chicks is also distinct from riboflavin. Birch, György and Harris ('35) found that the distribution of the rat dermatitis preventive factor in various cereals differed from the known distribution of the human pellagra preventive factor and Dann ('36) showed that rat dermatitis is etiologically different from human pellagra and that the human pellagra preventive factor is distinct from lactoflavin; it was thus demonstrated that vitamin B_2 comprises three distinct factors: riboflavin, the rat dermatitis preventive, and the human pellagra preventive factors. Next the separation of the rat dermatitis and the chick dermatitis

TABLE 4

The four sharply characterized components of the vitamin G complex

A. Factors which have been isolated and identified

Factor	*Property*	*Reference*
1. Riboflavin	Essential for growth of rat but not preventive of rat dermatitis.	György et al. ('33) György ('34)
	Essential for growth of chick.	Lepkovsky and Jukes ('35)
	Not preventive of chick dermatitis.	Elvehjem and Koehn ('34)
	Prevents dermatitis of turkey poults.	Lepkovsky and Jukes ('36)
	Prevents cataract in rats.	Day and Darby ('36)
	Prevents 'yellow liver' of dogs.	Sebrell et al. ('38)
	Does not cure blacktongue.	Sebrell et al. ('37)
	Does not cure pellagra.	Dann ('36) Ruffin and Smith ('37)
2. Nicotinic acid	Cures blacktongue.	Elvehjem et al. ('37) This paper
	Cures pellagra.	Fouts et al. ('37) Smith et al. ('37) Hassan and Harris ('37) Spies and Blankenhorn ('37)
	Does not cure rat dermatitis or prevent chick dermatitis.	This paper

B. Factors which have been distinguished but not isolated or identified

3. The rat dermatitis preventive factor (vitamin B_x)	By definition will prevent or cure rat dermatitis.	
	Is present in corn and therefore cannot prevent blacktongue or pellagra.	Birch et al. ('35) Dann ('36)
	Will not prevent chick dermatitis.	Lepkovsky, Jukes and Krause ('36)
4. The chick dermatitis preventive factor (filtrate factor of Lepkovsky and Jukes ('35), vitamin B_x of Elvehjem and coworkers ('34))	By definition will prevent chick dermatitis.	
	Will not prevent rat dermatitis, but probably essential for rat growth.	Lepkovsky, Jukes and Krause ('36)

preventive factors was achieved by Lepkovsky, Jukes and Krause ('36), and Jukes ('37) was also able to demonstrate that the chick dermatitis and human pellagra preventive factors are differently distributed in certain foodstuffs, suggesting that they are separate entities. The next steps forward were the finding of Elvehjem et al. ('37) that nicotinic acid will cure blacktongue in dogs and the subsequent demonstration by Fouts et al. ('37), Smith, Ruffin and Smith ('37), Harris and Hassan ('37) and Spies, Cooper and Blankenhorn ('37) that nicotinic acid will also cure human pellagra. The evidence put forward in this communication distinguishes the chick dermatitis preventive factor from nicotinic acid and affords final proof of the suggestion arising from the work of Jukes ('37); it follows that four members of the vitamin B_2 complex have been distinguished and separated one from another. These factors and their distinctive properties are listed in table 4.

It must be borne in mind that other factors may later be added to this list; future work alone can decide whether it is complete. Also the experiments which demonstrate that nicotinic acid will not cure the dermatitis of rats or chicks do not decide the question whether nicotinic acid is a dietary essential for either of these species. The mean figures in table 2B show an increased growth in chicks receiving nicotinic acid over the growth of the negative controls, but the weight increase of individual chicks of each group varies sufficiently so that the standard deviation of the means is so large that the difference between the means is not significant.

SUMMARY

Data are presented to show that nicotinic acid will not cure rat dermatitis or prevent chick dermatitis, and it is concluded that vitamin B_6 and the filtrate factor are both distinct from nicotinic acid.

The finding of Elvehjem et al. (loc. cit.) that nicotinic acid will cure blacktongue in dogs has been confirmed.

These results are regarded as affording direct evidence that the vitamin B_2 complex contains the following four different, sharply characterized entities: riboflavin, nicotinic acid, vitamin B_6 and the filtrate factor.

LITERATURE CITED

BIRCH, T. W., P. GYÖRGY AND L. J. HARRIS 1935 The vitamin B_2 complex. Differentiation of the anti-blacktongue and the 'P-P' factors from lactoflavin and vitamin B_6 (so-called 'rat pellagra' factor). Biochem. J., vol. 29, pp. 2830–2850.

COHN, E. J., G. R. MINOT, G. A. ALLES AND W. T. SALTER 1928 The nature of the material in liver effective in pernicious anemia. J. Biol. Chem., vol. 77, p. 325.

DANN, W. J. 1936 The vitamin G complex. I. The non-identity of rat dermatitis due to vitamin B_6 deficiency and the dermatitis of human pellagra. J. Nutrition, vol. 11, pp. 451–462.

———— 1937 Nicotinic acid and vitamin B_6. Science, vol. 86, pp. 616–617.

DAY, P. L., AND W. J. DARBY 1936 The inverse relation between growth and incidence of cataract in rats given graded amounts of vitamin G-containing foods. J. Nutrition, vol. 12, pp. 387–394.

ELVEHJEM, C. A., AND C. J. KOEHN 1934 Non-identity of vitamin B_2 and flavines. Nature, vol. 134, pp. 1007–1008.

ELVEHJEM, C. A., R. J. MADDEN, F. M. STRONG AND D. W. WOOLLEY 1937 Relation of nicotinic acid and nicotinic acid amide to canine blacktongue. J. Am. Chem. Soc., vol. 59, pp. 1767–1768.

FOUTS, P. J., O. M. HELMER, S. LEPKOVSKY AND T. H. JUKES 1937 Treatment of human pellagra with nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 37, pp. 405–407.

GOLDBERGER, J., AND R. D. LILLIE 1926 A note on an experimental pellagra-like condition in the albino rat. U. S. Pub. Health Rep., vol. 41, pp. 1025–1029.

GYÖRGY, P. 1934 Vitamin B_2 and the pellagra-like dermatitis in rats. Nature, vol. 133, pp. 498–499.

GYÖRGY, P., R. KUHN AND TH. WAGNER JAUREGG 1933 Das vitamin B_2. Naturwiss., Bd. 21, S. 560–561.

HARVEY, H. I., D. T. SMITH, E. L. PERSONS AND M. V. BURNS 1938 The effect of various fractions of liver on experimental canine blacktongue. J. Nutrition, vol. 16, p. 153.

HARRIS, L. J., AND A. HASSAN 1937 Quoted in Nature, vol. 140, p. 1070.

JUKES, T. H. 1937 Further observations on the assay, distribution and properties of the filtrate factor. J. Biol. Chem., vol. 117, pp. 11–20.

LEPKOVSKY, S., AND T. H. JUKES 1935 Growth promoting effect of flavine on the chick. Science, vol. 82, p. 326.

———— 1936 The response of rats, chicks and turkey poults to crystalline vitamin G (flavin). J. Nutrition, vol. 12, pp. 515–526.

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, pp. 557–566.

MCCREA, F. D., AND W. J. DANN 1937 Unpublished observations.

RUFFIN, J. M., AND D. T. SMITH 1937 A clinical evaluation of the potency of various extracts of liver in the treatment of pellagra. So. Med. J., vol. 30, pp. 4–14.

SEBRELL, W. H., D. J. HUNT AND R. H. ONSTOTT 1937 Lactoflavin in the treatment of canine blacktongue. U. S. Pub. Health Rep., vol. 52, pp. 235–239.

SEBRELL, W. H., AND R. H. ONSTOTT 1938 Riboflavin deficiency in dogs. U. S. Pub. Health Rep., vol. 53, pp. 83–94.

SMITH, D. T., J. M. RUFFIN AND S. G. SMITH 1937 Pellagra successfully treated with nicotinic acid: a case report. J. Am. Med. Assn., vol. 109, pp. 2054–2055.

SMITH, M. I., AND E. G. HENDRICK 1926 Some nutrition experiments with brewer's yeast. U. S. Pub. Health Rep., vol. 41, pp. 201–207.

SPIES, T. D., C. COOPER AND M. A. BLANKENHORN 1937 Quoted in J. Am. Med. Assn., vol. 110, p. 289.

SUBBAROW, Y., B. M. JACOBSON AND C. H. FISKE 1936 A partially purified liver extract therapeutically effective in pernicious anemia. New Eng. J. Med., vol. 214, p. 194.

SUBBAROW, Y., B. M. JACOBSON AND V. PROCHOWNICK 1936 Studies of the pernicious anemia principle in liver. III. The isolation and properties of a substance with primary therapeutic activity. J. Am. Chem. Soc., vol. 58, pp. 2234–2236.

UNDERHILL, F. P., AND L. B. MENDEL 1928 A dietary deficiency canine disease —further experiments on the diseased condition in dogs described as pellagra-like by Chittenden and Underhill and possibly related to so-called blacktongue. Am. J. Physiol., vol. 83, pp. 589–633.

PRODUCTION OF MICROCYTIC HYPOCHROMIC ANEMIA IN PUPPIES ON SYNTHETIC DIET DEFICIENT IN RAT ANTIDERMATITIS FACTOR (VITAMIN B_6)[1]

PAUL J. FOUTS, O. M. HELMER, S. LEPKOVSKY AND T. H. JUKES

Lilly Laboratories for Clinical Research, Indianapolis City Hospital, Indianapolis, and Division of Poultry Husbandry, University of California, Berkeley

(Received for publication February 9, 1938)

It is now recognized that the vitamin B_2 complex may be divided into at least four components: riboflavin, rat antidermatitis factor (vitamin B_6), chick antidermatitis factor (filtrate factor) and nicotinic acid (György, '35; Elvehjem and Koehn, '35; Hogan and Richardson, '35; Lepkovsky and Jukes, '36; Lepkovsky, Jukes and Krause, '36, and Elvehjem, Madden, Worley and Strong, '37).

It was the purpose of these studies to rear puppies on synthetic diets supplemented with vitamin B_1 (antineuritic vitamin) and the individual components of the vitamin B_2 complex and then to study the deficiencies produced by absence of the individual components.

Few studies have been reported on dogs maintained on synthetic diets. Birch, György and Harris ('35) produced blacktongue in dogs fed a purified diet supplemented with vitamin B_1 and riboflavin. Cure of the condition was effected only after the addition of both liver extract and maize to the diet. Street ('37) likewise produced blacktongue in dogs on a similar diet supplemented only with a vitamin B_1 concentrate. Zimmerman, Cowgill and Fox ('37) have recently reported the neurological findings in dogs on a purified diet supplemented with vitamin B_1 concentrate and minimal amounts of liver extract.

[1] Presented in part at the meeting of the American Society of Biological Chemists, Memphis, Tennessee, J. Biol. Chem., vol. 34, p. 117 (1937).

Deficiencies due to absence of the individual components of the vitamin B_2 complex have not been studied in dogs. However, the value of some of the fractions as regards their ability to cure blacktongue has been tested on dogs maintained on Goldberger diets. Koehn and Elvehjem ('36, '37) have cured blacktongue by administration of a highly purified liver extract containing chick antidermatitis (filtrate) factor. Riboflavin had no curative value. Booher and Hansmann ('36) also observed that a concentrate obtained from whey powder was effective in prevention and cure of blacktongue. In addition, since the completion of our studies, Elvehjem, Madden, Worley and Strong ('37) have cured blacktongue by administration of nicotinic acid and nicotinic acid amide. Street and Cowgill ('37) and Dann ('37) have confirmed these findings as to the cure of blacktongue with nicotinic acid.

This paper deals mainly with results of study of puppies maintained on a synthetic diet apparently deficient only in rat antidermatitis (vitamin B_6) factor.

MATERIALS AND METHODS

Mongrel puppies obtained soon after weaning were used. The basic diet consisted of the following: casein [2] 41.4 gm., sucrose 29.6 gm., Crisco 25.7 gm., bone ash 2.0 gm. and salt mixture no. 185 (McCollum and Simmonds, '18) 1.3 gm. Each puppy received 15 drops of halibut liver oil daily. In addition, they received various combinations of the following supplements: crystalline riboflavin, concentrated vitamin B (Stuart, Block and Cowgill, '34), thiamine (crystalline vitamin B), liver extract K-50 of Lepkovsky, Jukes and Krause ('36) or liver extract 1A[3] to supply chick antidermatitis (filtrate) factor and nicotinic acid, rice polish extracts K-48

[2] The casein was purified by dissolving in alkali and reprecipitating with acid. This procedure was repeated three times.

[3] Liver extract 1A was prepared from powdered liver extract (Lilly) by two adsorptions with Lloyd's reagent to remove vitamin B, riboflavin and rat antidermatitis factor. The filtrate was further purified by precipitation with alcohol and ether. The alcohol ether soluble extract was freed of alcohol and ether and concentrated so that 1 cc. of liver extract 1A was derived from 0.5 gm. of powdered liver extract.

(Lepkovsky, Jukes and Krause, '36) and K-87.D[4] to supply rat antidermatitis factor. Each of these supplements had been shown by tests on chicks and rats to be free from other known components of the vitamin B complex before they were administered. Nicotinic acid had not been separated from the chick antidermatitis factor at the time these studies were made, hence the extracts were not tested for it. However, our findings suggest that the concentrated vitamin B, crystalline vitamin B, riboflavin and the rice polish extracts were free from nicotinic acid since the puppies developed blacktongue while receiving these supplements. The liver extracts used are similar to the extract from which nicotinic acid was isolated.

The supplements were incorporated into a small portion of diet. After the dogs had eaten this they were allowed as much of the basal diet as desired. When anorexia developed the supplements were fed either by tube or dropper if the animals would not take them voluntarily.

RESULTS

Dogs 1 and 2 demonstrate that puppies can be reared on a purified casein diet properly supplemented. They received daily 0.2 mg. of crystalline riboflavin; 0.5 cc. concentrated vitamin B; 1.0 cc. liver extract K-50 per kilo; 0.5 cc. of rice polish extract K-48 per kilo and halibut liver oil. Dog 1 gained 7.6 kilos in 134 days and dog 2 gained 7 kilos in 121 days on the purified, supplemented diet. Their red blood-cell counts and hemoglobin were normal throughout the study.

Both of these dogs developed blacktongue when liver extract and rice polish extract were discontinued. The red cell count of dog 1 was 9.04 million and hemoglobin 20.3 gm. at the height of the symptoms of blacktongue, while that of dog 2 was 8.44 million and hemoglobin 17 gm. These high counts probably resulted from dehydration.

Dogs 3 and 4, who received all supplements except liver extract, died with symptoms of blacktongue. The diet of dog 5

[4] Rice polish K-87.D was prepared as K-48 except that it was heated in a boiling water bath at pH 10.3 for 3 hours.

was deficient in rice polish extract as well as liver extract. He, likewise, died with symptoms of blacktongue.

The basic diet of dogs 6 and 7 was supplemented with 0.5 cc. of concentrated vitamin B, 0.2 mg. riboflavin, liver extract K-50 1.0 cc. per kilo, and halibut liver oil. The gain in weight of dog 6 was not satisfactory. Convulsions occurred occasionally after the 80th day. By the 100th day of study the red blood count was 2.16 million and hemoglobin 2.5 gm. Reticulocytes increased in number (10.2%) following iron therapy (5 grains of ferrous sulphate daily) but the red blood cells decreased. By the 109th day the red cell count was 1.83 million, hemoglobin 1.4 gm., color index 0.25, and mean diameter of the red blood cells 4.6 μ. Death occurred on the 112th day of the experiment. Autopsy showed all tissues to be pale. The spleen was enlarged and quite soft. The liver was filled with more than usual amounts of fat. The bone marrow from the mid-portion of the femur was red and hyperplastic. The majority of the nucleated red blood cells was normoblasts.

Dog 7 gained in weight until the 97th day. By the 175th day the red cell count was 2.93 million, hemoglobin 2.8 gm., color index 0.22, and mean diameter of the red blood cells 4.9 μ. The white cell count was 15,550.

Beginning about the 138th day the dog developed involuntary twitching of the muscles. These varied in intensity and occasionally there were generalized convulsions. Blood sugar, serum calcium, blood urea nitrogen and serum protein determinations were made, but these were normal and thus did not help to explain the convulsions.

Rice polish adsorbate administered daily from the 175th day was followed by a reticulocyte rise up to 34.5%. In spite of rapid rise in red blood cells and hemoglobin and temporary improvement in weight and general condition, the convulsions increased in severity and frequency. She died in a convulsion on the 190th day. The red blood-cell count was 7.3 million and the hemoglobin 13.1 gm. on the 187th day. Bone marrow appeared normal at post-mortem.

TABLE 1

Response of weight, red blood count, hemoglobin, reticulocytes and white blood count of dog 8 to diet deficient in rat antidermatitis factor and subsequent response to rice polish extract apparently containing only this factor

Days on diet	*Weight in kilos*	*RBC in million per cubic millimeter*	*HGB in grams per 100 cc. (Newcomer)*	*WBC per cubic millimeter*	*Reticulocyte percentage*	
1	3.9	5.52	11.5	20,300		
18	4.5	5.62	12.3	12,250		
31	4.6	7.64	15.3	14,000		
45	4.7	6.93	13.6	11,200		
59	5.0	7.13	13.4	11,900		
73	5.2	6.68	12.3	9,050		
87	5.2	6.37	10.0	10,700		
101	5.2	5.07	8.0	14,100		
108	4.9	4.97	7.5	14,700		
115	5.0	4.97	7.3	9,050		
122	5.2	4.30	5.8	9,200		
127	5.0	4.26	6.7	8,150		
129	5.2	4.58	7.2	13,850		
134	5.1	5.15	8.3	11,200		
136	5.1	4.93	7.5	9,250		
141	5.2	4.79	6.7	12,350		
143	5.2	4.76	6.6	8,550		
161	5.1	3.47	4.4	11,750	0.6	
162	5.2				1.1	
163	5.2				0.6	
164	5.1	2.78	3.6	9,700	0.7	—Rice polish K-87.D 0.2 cc. per kilo daily
165	5.0				1.2	
166	5.2				2.2	
167					16.4	
168	5.2	4.06	7.0	9,400	14.9	
169	5.4				2.0	
170	5.5				2.0	
171	5.5	4.84	8.7	12,300	0.2	
176	5.9	5.11	11.5	14,100		
178	6.1	5.42	11.9			
183	6.4	5.70	11.9	18,300		
185	6.5	5.73	11.9	14,850		
192	6.8	5.75	12.8	11,600		
199	7.1	6.24	13.4	15,900		
206	7.7	6.96	13.9	18,450		
213	7.6	6.90	13.7	16,250		
220	8.1	7.84	14.7	17,900		

TABLE 2

Response of weight, red blood count, hemoglobin, reticulocytes and white blood count of dog 9 to diet deficient in rat antidermatitis factor and subsequent response to rice polish extract apparently containing only this factor

Days on diet	*Weight in kilos*	*RBC in million per cubic millimeter*	*HGB in grams per 100 cc. (Newcomer)*	*WBC per cubic millimeter*	*Reticulocyte percentage*
1	3.9	4.95	11.1	21,150	
18	4.8	5.67	12.5	18,900	
31	5.1	6.29	12.6	21,350	
45	5.3	5.97	11.4	18,300	
59	5.4	5.26	9.8	14,250	
73	5.5	4.94	8.4	12,350	
80	5.5	4.10	6.3	12,950	
87	5.4	3.56	5.6	14,450	
94	5.2	3.28	4.7	8,750	
101	5.2	2.68	3.3	16,450	1.0
102	5.2				0.5
105	5.2				0.5
106	5.1	2.32	2.6	14,700	0.5—Rice polish extract K-87.D 0.1 cc. per kilo daily
107	5.1				2.3
108	5.3	3.14	5.2	21,250	21.4
109	5.5				20.7
110	5.8	4.34	8.0		7.5
111					6.0
112	5.6	4.91	8.7	11,800	5.4
113	5.9	4.70	8.6	11,650	
114	6.1				2.0
115	6.2	4.98	9.4	14,150	1.4
120	6.7	5.26	9.7	13,950	
122	6.8	5.17	10.8	17,800	
127	6.8	4.82	10.3	24,100	0.4
128	7.0				1.0
129	7.2	4.95	10.9	20,350	0.2
130	7.2				0.2—Rice polish extract K-87.D 0.2 cc. per kilo daily
131	7.3				2.3
132	7.4				2.4
133					2.0
134	7.5	5.19	11.7	25,250	0.6
136	7.3	5.35	11.7	20,800	0.2
141	7.3	5.94	13.1	12,050	
143	7.4	5.87	13.1	11,800	
161	8.1	6.17	13.0	13,200	
163	7.8	6.26	13.1	11,150	
171	8.1	6.37	13.7	11,950	
178	8.2	6.64	14.4		

The diet of dogs 8 and 9 was supplemented with 0.5 mg. of thiamine per kilo, 60 micrograms of riboflavin per kilo, 1.0 cc. of liver extract 1A per kilo (2 cc. per kilo after the thirty-first day), and 15 drops of halibut liver oil. Their weights did not increase at normal rate (tables 1 and 2). Both developed a severe microcytic hypochromic anemia. By the 164th day the red blood count of dog 8 had decreased from 7.64 to 2.78 million, hemoglobin from 15.3 to 3.6 gm., and mean diameter of the red blood cells from 6.6 to 5.7 μ. The white blood-cell count was 9700 and the color index 0.41. The red blood count of dog 9 decreased steadily from 6.29 to 2.32 million by the 106th day. Hemoglobin decreased from 12.6 to 2.6 gm. and mean diameter of the red blood cells decreased from 6.9 to 4.6 μ. White blood count was 14,700 and blood non-protein nitrogen, blood urea nitrogen and serum proteins were normal on the 106th day. Dog 8 had three convulsions while anemic.

Rice polish extract K-87.D was then administered to both dogs. Reticulocyte peaks of 16.4% and 21.4% (tables 1 and 2) were followed by rapid rises in hemoglobin and red blood-cell counts. Their weights which had been stationary then increased. The red blood count and hemoglobin of dog 9, however, did not reach normal values until after the dose of rice polish extract was increased to 0.2 cc. per kilo. A second rise (2.4%) in reticulocytes followed this increase in rice polish extract.

DISCUSSION

These observations show that it is possible to raise puppies on a purified diet supplemented with concentrated vitamin B or thiamine, riboflavin, purified liver extract containing chick antidermatitis (filtrate) factor and nicotinic acid but free of rat antidermatitis factor and riboflavin, rice polish extract containing rat antidermatitis factor but no chick antidermatitis factor or riboflavin, and vitamins A and D.

In the four dogs receiving all supplements except rice polish extract containing rat antidermatitis factor, severe anemia (hemoglobin 1.4, 2.8, 3.6 and 2.6 gm.) developed. Iron failed to cure the anemia of one dog to which it was administered.

Addition of rice polish extract containing rat antidermatitis factor to diet of three dogs was followed by rise in reticulocytes and rapid disappearance of anemia. The peaks of the reticulocyte responses were reached on the second or third day of therapy. They were thus from 3 to 7 days earlier than those that follow administration of liver extract to pernicious anemia patients, or iron to patients with iron deficiency anemias. In addition, the increase in hemoglobin and red blood cells was rapid considering the fact that at the time of these increases there was also a rapid gain in weight and therefore in blood volume.

Microcytic hypochromic anemia did not develop in any of the nineteen dogs studied except in the four dogs receiving diets deficient only in rice polish extract. The dogs on diets deficient in both rice polish extract and liver extract died with symptoms of blacktongue before anemia developed. At no time were there symptoms of blacktongue associated with uncomplicated deficiency of the material contained in rice polish extract.

Anemia associated with deficiencies in the B vitamins has been reported (Whitehead and Barlow, '29; Sure, Kik and Smith, '31; Rhoads and Miller, '33; Spies and Dowling, '35; Miller and Rhoads, '35; Day, Langston and Shukers, '35; Wells and Stewart, '35, and Hogan, Richardson and Johnson, '37) although Rose and Stucky ('28), Cartland and Koch ('28) and Zimmerman, Cowgill and Fox ('37) failed to find it. The variation in development and character of the anemia might be explained on the fact that these investigators were studying deficiencies of different components or combinations of components of the B vitamins. Although there is no information to the contrary, present knowledge does not justify the conclusion that the material in rice polish extract which cures rat dermatitis is identical with the substance that cures anemia in dogs. Since completion of our studies, György, Goldblatt, Miller and Fulton ('37) have reported the development in rats of panmyelophthisis with hemorrhagic manifestations.

This condition developed during vitamin B_6 deficiency studies, but György ('38) has been able to prevent its occurrence by inclusion of nicotinic acid in vitamin B_6 deficient diet.

Blacktongue did not develop in puppies fed diet supplemented with liver extract K-48 and 1A. These liver extracts contained chick antidermatitis (filtrate) factor. Elvehjem, Madden, Worley and Strong ('37), however, have recently observed that nicotinic acid and nicotinic acid amide cured blacktongue. They first isolated nicotinic acid from a purified liver extract containing chick antidermatitis factor and similar to the liver extracts used in studies reported in this paper. Lepkovsky and Jukes (unpublished data), Dann ('37) and Cook, Clarke and Light ('37) have been unable to substitute nicotinic acid either for the chick antidermatitis (filtrate) factor or for the rat antidermatitis (vitamin B_6) factor under their experimental conditions. It would therefore appear that the purified liver extract contains at least two active substances, one of which (chick antidermatitis factor) will prevent pellagra-like symptoms in chicks, and the other (nicotinic acid) prevent and cure blacktongue in dogs and cure pellagra in humans (Fouts, Lepkovsky, Helmer and Jukes, '36, '37; Spies, Cooper and Blankenhorn, '37, and Smith, Ruffin and Smith, '37).

SUMMARY

1. Puppies showed normal rate of growth on purified casein diets, supplemented with concentrated vitamin B or thiamine, crystalline riboflavin, liver extract containing the chick antidermatitis (filtrate) factor and nicotinic acid but free from both riboflavin and the rat antidermatitis factor, and rice polish extract containing the rat antidermatitis factor (vitamin B_6) free of the chick antidermatitis factor and riboflavin.

2. Severe microcytic hypochromic anemia developed in the puppies when rat antidermatitis (vitamin B_6) factor was apparently the only missing component of the diet. This anemia was cured by addition to the diet of this missing factor.

3. Blacktongue was produced in the puppies whenever the liver extract containing nicotinic acid and chick antidermatitis (filtrate) factor was absent from the diet.

LITERATURE CITED

BIRCH, T. W., P. GYÖRGY AND L. J. HARRIS 1935 The vitamin B_2 complex. Differentiation of the anti-blacktongue and 'P-P' factors from lactoflavin and vitamin B_6 (so-called 'rat pellagra' factor). Parts I–VI. Biochem. J., vol. 29, p. 2830.

BOOHER, L. E., AND G. H. HANSMANN 1936 Vitamin G concentrates as preventives against blacktongue, with parallel studies of same concentrates in diets of white rats. Am. J. Physiol., vol. 114, p. 429.

CARTLAND, G. F., AND F. C. KOCH 1928 Studies on hemoglobin formation in rats. Proc. Soc. Exp. Biol. and Med., vol. 25, p. 447.

COOK, C. A., M. R. CLARKE AND A. E. LIGHT 1937 Relation of nicotinic acid to growth and dermatitis factor in rice polishings. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 514.

DANN, W. J. 1937 Nicotinic acid and vitamin B_2. Science, vol. 86, p. 616.

DAY, P. L., W. C. LANGSTON AND C. F. SHUKERS 1935 Leukopenia and anemia in the monkey resulting from vitamin deficiency. J. Nutrition, vol. 9, p. 637.

ELVEHJEM, C. A., AND C. J. KOEHN, JR. 1935 The non-identity of vitamin B_1 and flavins. J. Biol. Chem., vol. 108, p. 709.

ELVEHJEM, C. A., R. J. MADDEN, D. W. WORLEY AND F. M. STRONG 1937 Relation of nicotinic acid and nicotinic acid amide to canine blacktongue. J. Am. Chem. Soc., vol. 59, p. 1767.

FOUTS, P. J., S. LEPKOVSKY, O. M. HELMER AND T. H. JUKES 1936 Successful treatment of human pellagra with the 'filtrate factor.' Proc. Soc. Exp. Biol. and Med., vol. 35, p. 245.

FOUTS, P. J., O. M. HELMER, S. LEPKOVSKY AND T. H. JUKES 1937 Treatment of human pellagra with nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 405.

GYÖRGY, P. 1935 Investigations on the vitamin B_2 complex. I. The differentiation of lactoflavin and the 'rat antipellagra' factor. Biochem J., vol. 29, p. 741.

———— 1938 Nicotinic acid and prevention of nutritional panmyelophthisis in rats. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 732.

GYÖRGY, P., H. GOLDBLATT, F. R. MILLER AND R. P. FULTON 1937 Panmyelophthisis with hemorrhagic manifestations in rats on a nutritional basis. J. Exp. Med., vol. 66, p. 579.

HOGAN, A. G., AND L. R. RICHARDSON 1935 Plural nature of vitamin B. Nature, vol. 136, p. 186.

HOGAN, A. G., L. R. RICHARDSON AND P. E. JOHNSON 1937 A new deficiency disease associated with the vitamin B complex. Proc. Am. Soc. Biol. Chem., J. Biol. Chem., vol. 117, p. L.

JUKES, T. H., AND S. LEPKOVSKY 1936 The distribution of the filtrate factor (a water-soluble vitamin belonging to the vitamin B complex and preventing a dietary dermatitis in chicks) in certain feeding stuffs. J. Biol. Chem., vol. 114, p. 117.

KOEHN, C. J., JR., AND C. A. ELVEHJEM 1936 Studies on vitamin G (B_2) and its relation to canine blacktongue. J. Nutrition, vol. 11, p. 67.

——— 1937 Further studies on the concentration of the antipellagra factor. J. Biol. Chem., vol. 118, p. 693.

LEPKOVSKY, S., AND T. H. JUKES 1936 The effect of some reagents on the 'filtrate factor' (a water-soluble vitamin belonging to the vitamin B complex and preventing a dietary dermatitis in chicks). J. Biol. Chem., vol. 114, p. 109.

——— Unpublished data.

LEPKOVSKY, S., T. H. JUKES AND M. E. KRAUSE 1936 The multiple nature of the third factor of the vitamin B complex. J. Biol. Chem., vol. 115, p. 557.

MCCOLLUM, E. G., AND N. SIMMONDS 1918 A study of the dietary essential, water-soluble B, in relation to its solubility and stability toward reagents. J. Biol. Chem., vol. 33, p. 55.

MILLER, D. K., AND C. P. RHOADS 1935 The experimental production of loss of hematopoietic elements of the gastric secretion and of the liver in swine with achlorhydria and anemia. J. Clin. Invest., vol. 14, p. 153.

RHOADS, C. P., AND D. K. MILLER 1933 The production in dogs of chronic blacktongue with anemia. J. Exp. Med., vol. 58, p. 585.

ROSE, W. B., AND C. J. STUCKY 1928 Anhydraemia in rats suffering from lack of what has been called vitamin B. Proc. Soc. Exp. Biol. and Med., vol. 25, p. 687.

SMITH, T. D., J. M. RUFFIN AND S. G. SMITH 1937 Pellagra successfully treated with nicotinic acid. J. Am. Med. Assoc., vol. 109, p. 2054.

SPIES, T. D., C. COOPER AND M. A. BLANKENHORN 1937 Therapeutic administration of nicotinic acid in human beings during health and disease. Central. Soc. for Clin. Research, Chicago, November 5, 1937.

SPIES, T. D., AND A. S. DOWLING 1935 The experimental production of anemia in dogs by means of a blacktongue-producing diet. Am. J. Physiol., vol. 114, p. 25.

STREET, H. R. 1937 Production of canine blacktongue on purified diets. Proc. Soc. Exp. Biol. and Med., vol. 36, p. 602.

STREET, H. R., AND G. R. COWGILL 1937 The cure of canine blacktongue with nicotinic acid. Proc. Soc. Exp. Biol. and Med., vol. 37, p. 547.

STUART, E. H., R. J. BLOCK AND G. R. COWGILL 1934 The antineuritic vitamin. V. The preparation of a vitamin concentrate suitable for parenteral use. J. Biol. Chem., vol. 105, p. 463.

SURE, B., M. C. KIK AND M. E. SMITH 1931 Hematopoietic function in avitaminosis. VI. Vitamin G deficiency. Proc. Soc. Exp. Biol. and Med., vol. 28, p. 498.

WHITEHEAD, R. W., AND O. W. BARLOW 1929 Study of anemia of rice disease in rats. The influence of vitamins A, B, D, iron, copper, beef muscle, and liver on the course and regeneration from the anemia of rice disease. Am. J. Physiol., vol. 89, p. 542.

WELLS, L., AND A. STEWART 1935 Experimental anemia in monkeys, with special reference to macrocytic nutritional anemia. Brit. J. Exp. Path., vol. 16, p. 444.

ZIMMERMAN, H. M., G. R. COWGILL AND J. C. FOX, JR. 1937 Neurologic manifestations in vitamin G (B_2) deficiency. Arch. Neur., vol. 37, p. 286.